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(54) Title: GLIAL ANTIPROLIFERATIVE PROTEINS

(57) Abstract

Glial antiproliferative proteins comprising a neural antiprolifertive protein (NAP) of about 55kD produced by glial cells and having a metalloprotease activity, and cryptic antiproliferative fibronectin fragments (CAFF) comprising those fibronectin fragments generated by action of the NAP protease on fibronectin and having the property of inhibiting the growth of glial cells. The NAP and CAFF glial antiproliferative proteins are useful in promoting regeneration of nervous tissue following trauma of injury or surgery, and in retarding the growth of glial tumors. Monoclonal antibodies to the glial antiproliferative proteins are useful in the treatment of demyelinating diseases, such as multiple sclerosis.

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GLIAL ANTIPROLIFERATIVE PROTEINS

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Background of the Invention

The invention relates to the regulation of growth and development of tissues of the nervous system and particularly to agents capable of modulating proliferation of cells of the nervous system.

During development of an animal, each of the major glial cell types proliferate in response to as yet undefined mitogens which are present within their tissue environment. After a defined period, the cells quit proliferating, differentiate and remain in a relatively quiescent, non-dividing state throughout normal adulthood. However, when nerve tissue is diseased or damaged, normal glial proliferation may be decreased producing a hypoplastic tissue or may increase unchecked to produce a hyperplastic tissue. In yet other cases cells may become transformed and proliferate wildly to form tumors. In any case, an abnormal number of glial cells may impede tissue repair.

The three major glial cell types are Schwann cells in the peripheral nervous system (PNS) and oligodendroglial and astroglial cells in the central nervous system (CNS). Each glial cell type populates its corresponding tissue environment at specific times during development and then becomes relatively quiescent (non-dividing) after development. The following descriptions represent a review of these three glial cell types with respect to what is known about their proliferation.

During the development of the peripheral nervous system, Schwann cells populate peripheral nerve fibers apparently in response to contact with a mitogenic signal present on the axonal surface (DeCoster, M. and DeVries, G., J. Neurosci. Res. 22:283-288 (1989); Salzer and Bunge, J. Cell Biol., 84, 739-752 (1980a), 84, 753-766 (1980b), 84, 767-778 (1980c);

Wood and Bunge, Nature, 256, 661-664 (1975)). Contact and ensheathment of axons is required for subsequent Schwann cell differentiation involving the formation of a basal lamina, further axonal ensheathment and ultimately, myelination (Bunge and Bunge, J. Cell. Biol., 78, 943-950 (1978); Eldridge, et al., J. Neurosci., 9, 625-38 (1989); Williams, et al., J. Cell Biol., 70, 138-153 (1976)). After differentiation and in the healthy adult peripheral nerve, Schwann cells are believed to be relatively quiescent. This includes myelinating, ensheathing, perinodal, periaxonal and ganglionic Schwann cells.

The mature peripheral nerve characteristically consists of axons wrapped by Schwann cells and surrounded by a basal lamina containing laminin, collagen, fibronectin and heparin sulfate proteoglycan, which in turn is itself embedded in a collagenous matrix. The overall nerve structure is relatively stable in the sense that Schwann cells undergo little, if any, proliferation and axonal sprouting and growth are apparently suppressed. However, these quiescent properties are not irreversibly imposed since axonal disruption results in Wallerian degeneration involving reentry of Schwann cells into the mitotic cycle and active axonal regeneration (Payer, J. Comp. Neurol, 183, 365-383 (1979); Weinberg and Spencer, J. Neurocytol., 7, 555-569 (1978)). Because of their known abilities to promote cellular differentiation, extracellular matrix and associated molecules are likely candidates as growth regulating signals for cells in peripheral nerve (Bunge and Bunge, J. Cell. Biol., 78, 943-950 (1978); Bunge and Wood, Prog. Brain Res., 71, 143-52 (1987); Sanes, Ann. Rev. Neurosci., 12, 491-516 (1989)).

In addition to the neuritic mitogen, a limited array of soluble agents stimulate the proliferation of cultured Schwann cells (for review see Ratner, et al., Ann. N.Y. Acad. Sci., 486, 170-181 (1986) such as glial growth factor (Brockes, et al., J. Biol. Chem., 255, 8374-8377 (1980)), glial maturation factor (Bosch, et al., J. Neurosci., 9, 3690-3698 (1989)) transforming growth factor-ß or TGF-ß (Ridley, et al., J. Cell

Biol. 34:19-24 (1989), laminin and fibronectin (McGarvey, et al., Dev. Biol., 105, 18-28 (1984)), as well as agents such as cholera toxin and dibutyryl cyclic AMP that raise intracellular cAMP levels Brockes, et al., Brain Res., 165, 105-118 (1979). In addition, several autocrine mitogens have been postulated for Schwann cells including the basal lamina component laminin (McGarvey, et al., Dev. Biol., 105, 18-28 (1984) Bunge, et al., Annu. Rev. Neurosci., 9, 305-28 (1986) Muir, et al., Neurochem. Res., 14, 1013-1016 (1989b), Muir and Manthorpe, submitted, appended, (1990b)), glial maturation factor (Bosch, et al., Brain Res. 304:311-319 (1984)); Lim, et al., Dev. Brain Res., 40, 277-284 (1988)), TGF-β (Ridley, et al., J. Cell. Biol., 34, 19-24 (1989)) and an activity reported (Porter, et al., Proc. Natl. Acad. Sci. USA, 84, 7768-72 (1987)) in medium conditioned by primary and immortalized Schwann cells. Thus, cultured Schwann cells can produce a number of potential autocrine mitogens, and yet they remain virtually quiescent.

Oligodendroglial cells are the myelin-producing Schwann cell counterparts in the CNS and they also populate axons apparently in response to a mitogen present on the axolema (Bottenstein, et al., J. Neurosci. Res., 20, 291-303 (1988); Chen, S. and DeVries, G. J. Neurochem. 52:325-327 (1987); Sensenbrenner, et al., Journal De Physiologie , 82, 288-90 (1987)). Like Schwann cells, oligodendroglial cells appear to remain relatively quiescent after differentiation (Bottenstein, et al., J. Neurosci. Res., 20, 291-303 (1988); Ludwin and Bakker, 1988; Raff, M. Science 243:1450-1455 (1989)) and they are relatively quiescent in standard cell culture media. In vitro, oligodendroglial cells proliferate in response to fibroblast growth factor (Besnard, et al., Intl. J. Deve. Neurosci., 7, 401-9 (1989); Eccleston and Silberberg, Dev. Brain Res., 21, 315-318 (1985); Yong, et al., J. Cell. Biol., 104, 655-660 (1988)), platelet-derived growth factor (Besnard, et al., Neurosci. Lett., 73, 287-292 (1987)), interleukin-2 (Benveniste and Merrill, Nature, 321, 610-613 (1986); Suzumura and Silberberg, Brain Res., 480, 51-7

(1989)), insulin-like growth factor (McMorris and Dubois-Dalcq, J. Neurosci. Res., 21, 199-209 (1988)) as well as to less defined materials (Bologa, et al., J. Neurosci. Res., 20, 182-8 (1988); Hantaz-Ambroise, et al., Int. J. Dev. Neurosci., 6, 289-99 (1988); Yong, et al., J. Cell. Biol., 104, 655-660 (1988); Hunter and Bottenstein, Dev. Brain Res., 49, 33-49 (1989)). Oligodendroglial cell differentiation and myelination occur after proliferation and are influenced by interleukin-2 (Benveniste, et al., J. Neurochem., 49, 1274-9 (1987); Knobler, et al., Annals New York Acad. Sci., 540, 324-6 (1988); Suzumura and Silberberg, Brain Res., 480, 51-7 (1989)), insulin-like growth factor (McMorris and Dubois-Dalcq, J. Neurosci. Res., 21, 199-209 (1988)), platelet-derived growth factor (Anderson, 1989; Pringle, et al., EMBO Journal, 8, 1049-56 (1989)), ciliary neuronotrophic factor (Anderson, 1989), cyclic AMP (Riable and McMorris, Dev. Biol., 133, 437-46 (1989)), as well as less defined substances (Aloisi, F. et al., Neurochemica. Research 12: 189-195 (1988)); Bologa, L. et al., J. Neurosci. Res., 20, 182-8 (1988)). Thus, as with Schwann cells, oligodendroglial cell proliferation and differentiation may be coordinately promoted under a variety of stimuli.

During mammalian brain and spinal cord development, astroglial cells are generated during a specific period that occurs well after the formation of neurons but preceding the generation of oligodendroglial cells (for reviews, see Astrocytes, Vols. 1, 2, 3: S. Federoff and A. Vernadakis, eds.: 1986; Academic Press, Inc. NY). The exact mitogen(s) that stimulate their division in vivo is unknown. In vitro, purified rat astroglial cells replicate under the influence of a number of mitogens such as fibroblast growth factors (Perraud, et al., Glia 1:124-131 (1988)), platelet-derived growth factor (Besnard et al., Neurosci. Letters 73:287-292 (1987)), proteases such as thrombin (Perraud et al., Inter. J. Dev.Sci. 5:181-188 (1987)), protease inhibitors (Perraud et al., Int. J. Dev. Neurosci. 6:261-266 (1988)).

After cell proliferation ceases in vivo, glial cells remain in a relatively quiescent state in the adult. However, in response to nervous system damage or disease, glial cell proliferation can resume to produce a local excess of glial cells. In the PNS, Schwann cells proliferate during Wallerian degeneration to produce tightly packed rows of cells called "Bands of Bungner" which apparently provide growth avenues for incoming regenerating axons. In the CNS, astroglial cells proliferate around lesion areas to form a wall called the "glial scar" that may interfere with subsequent axon regeneration.

Cell proliferation in response to one factor can, in certain instances, be opposed by the addition of another factor (Wang and Hsu, Trends Biochem. Sci., 11, 24-26 (1986); Miyazaki and Horio, In Vitro Cell. Dev. Biol., 25, 866-872 (1989)). Currently about a dozen factors have been described that inhibit the proliferation of mitotically active cells. For example, interferon-β inhibits HeLa cells (Stewart, Springer-Verlag, Amsterdam, Netherlands (1979)), fibroblast growth factor (FGF) inhibits Ewing's human sarcoma cells (Schweigerer, et al., J. Clin. Invest. 80:1516-1520 (1987)), fibroblast growth inhibitor inhibits mouse embryo fibroblasts (Hsu and Wang, J. Cell. Biol., 102, 362-369 (1986)) and TGF-β (Tucker, et al., Science, 226, 705-707 (1984)) and BSC-1 growth inhibitor (Holley, et al., Proc. Natl. Acad. Sci. USA, 77, 5989-5992 (1980)) inhibit a wide variety of cell types. Some factors such as TGF-β can act as a mitogen for some cells but inhibit the proliferation of others (Bryckaert, et al., Exp. Cell Res., 179, 311-321 (1988)). All such antiproliferative factors appear to stop the cell cycle within the Go or G1 phases and cell proliferation can resume when the factor is removed. Thus far, no factors have been identified that irreversibly inhibit cell proliferation. Many of the above antiproliferative factors also affect other cell behaviors beside proliferation, usually by promoting processes associated with their normal differentiation.

Both Schwann and oligodendroglial cell proliferations apparently cease after the glial cells have saturated the available axonal surface (Bunge and Wood, Prog. Brain Res., 71, 143-52 (1987); Eccleston, et al., Exp. Cell Res., 182, 173-85 (1989b); Eldridge, et al., J. Neurosci., 9, 625-38 (1989); Manthorpe, et al., Brain Res., 196, 467-482 (1980)). It is conceivable that glial cell proliferation stops because of a loss of the neuronal mitogenic activity itself or a loss of glial responsiveness to the mitogen. Alternatively, glial cell proliferation in response to the neuronal mitogen may cease due to a new availability of antiproliferative activity or to the development of new glial cell responses to an antiproliferative activity. In vitro, non-glial cells appear to express a substance that inhibits the proliferation of astroblasts (Hatten, et al., J. Cell Biol., 104, 1353-60 (1987); Hatten and Shelanski, J. Neurosci, 8, 1447-53 (1988); Rogister, B. et al., J. Neuroscience Res. 25:58-70 (1990)) or Schwann cells (Eccleston, et al., Development, 107, 107-112 (1989a)) and interleukin-2 is reported to inhibit oligodendroglial precursor cell proliferation (Knobler, et al., Annals New York Acad. Sci., 540, 324-6 (1988); Saneto, et al., J. Neurosci. Res., 18, 147-54 (1987)).

Rat astroglial cell proliferation in vitro is also inhibited by a 17 kD protein called "Glia maturation factor" (Lim et al., Cancer Research 46:5241-5247 (1986)), an unidentified preparation entitled "glial growth inhibitory factor" (Kato and Tanaka, Brain Research 430:153-156 (1987)), and inhibitors of sterol biosynthesis such as mevinolin (Langan and Volpe, J. Neurochem. 46:1283-1291 (1986)).

Once cell proliferation ceases, Schwann and oligodendroglial cell differentiation and myelination occur and these processes are apparently regulated in a coordinate way by axons (Bosch, et al., J. Neurosci., 9, 3690-3698 (1989); Mokuno, et al., 1989; Williams, et al., J. Cell Biol., 70, 138-153 (1976)), basal lamina (Eldridge, et al., J. Neurosci., 9, 625-38 (1989)) and cell surface (Bunge and Bunge, J. Cell. Biol., 78, 943-950 (1978); Ranscht, et al., J.

Neurosci., 7, 2936-47 (1987)) components. An identification and characterization of glial cell antiproliferative factors could be an important step in advancing our understanding of regulatory components in the proliferation and subsequent differentiation of the myelinating glia.

It would be useful to identify and isolate growth regulating signals of glial cells. These agents could then be used to treat injuries and diseases caused by the abnormal growth and proliferation of normal glial cells and glial tumor cells.

It is therefore an object of the invention to provide antiproliferative agents that inhibit the growth of glial cells together with methods for the use of such agents.

Summary of the Invention

According to one aspect of the invention there is provided a composition consisting essentially of an effective, glial cell proliferation-inhibiting concentration of a glial antiproliferative protein, wherein said protein has protease activity. The invention further provides a pharmaceutical preparation, comprising an effective amount of the glial antiproliferative protein in a pharmaceutically acceptable carrier. The glial antiproliferative protein is a glial cell autocrine protein which is a calcium-dependent, Zn^{++}-containing protease isolated from glial cell culture conditioned media. According to another aspect of the invention this is further provided a method of isolating a peptide fragment from fibronectin, wherein the sequence has neural antiproliferative properties, comprising contacting fibronectin with the neural antiproliferative protease. The invention includes polypeptide sequences isolated from fibronectin by the method, and having a molecular weight of approximately 30 kD, as well as polypeptide sequences comprising at least some of the same amino acid sequences as the fibronectin fragment.

According to another aspect of the invention the neural antiproliferative protease and the glial antiproliferative fibronectin fragments may be prepared for pharmaceutical use by incorporation into suitable carriers or vehicles.

Preferred carriers are bio-erodable matrices, which may be implanted at the site of neural lesions. The bioeroda ble matrix comprising a neural antiproliferative agent provides a controlled release of the agent to the lesion.

According to yet another aspect of the invention there are provided methods of treating disease, employing the antiproliferative agents of the invention or antibodies thereto. In one embodiment of this aspect of the invention there is provided a method for inhibiting the proliferation of glial cells, comprising administering to the cells an effective proliferation-inhibiting amount of a glial antiproliferative protease or glial antiproliferative fibronectin fragment. In a preferred embodiment, the glial cells are in vivo. The glial cells may be associated with injured nervous system tissue, or alternatively the glial cells may be those of a tumor, such as a glioblastoma. In yet another embodiment of this aspect of the invention there is provided a method of preventing glial scarring following injury to nervous tissue in a patient, comprising applying to the neural lesion of the injury an effective glial cell proliferation-inhibiting amount of a glial antiproliferative protease or a glial antiproliferative fibronectin fragment. The methods of the invention may be applied in treating injuries of the nervous tissue or glial cell tumors in either the peripheral nervous system of the central nervous system.

According to yet another aspect of the invention, there are provided monoclonal antibodies having a specificity for at least a portion of the amino acid sequence of the neural antiproliferative protease or the neural antiproliferative fibronectin fragment of the invention. In a preferred embodiment of this aspect of the invention there is provided a method of treating demyelinating disease in a patient in need of such treatment, comprising administering an effective glial cell proliferation promoting amount of antibodies to a glial antiproliferative protein to the affected demyelinated lesions of the patient.

Brief Description of the Figures

Figure 1 shows the effects of cholera toxin and medium changes on Schwann cell proliferation.

Figure 2 shows the Schwann cell response to mitogens and its inhibition by Schwann cell conditioned media.

2A: inhibition titration of Schwann cell conditioned media

2B: BrdU staining of untreated cultures

2C: BrdU staining of treated cultures

Figure 3 shows the reversibility of the antiproliferative activity of Schwann cell conditioned media.

Figure 4 shows the response of transformed SC types to the antiproliferative activity.

Figure 5 is a set of chromatography elution profiles, as follows:

5A: CL4B chromatography of Schwann cell conditioned media.

5B: CL4B chromatography of RN22 Schwannoma conditioned media.

5C: CL4B chromatography of 4M urea-dissociated RN 22 1000 kD fractions.

5D: SDS-PAGE of 55 kD NAP activity purified from RN22 1000 kD material.

Figure 6 is an inhibition titration of a 30 kd fibronectin fragment.

Figure 7 is a schematic representation of the autocrine regulation of Schwann cell proliferation.

Figure 8 demonstrates the casein-degrading activity and anti-stromelysin immunoreactivity associated with the 55kD NAP.

Figure 9 shows the generation of proteolytic fibronectin (FN) fragments by the SC-derived 55 kD metalloprotease.

Figure 10 shows an antiproliferative activity in gel filtration fractions of a proteolytic mixture of the 55 kD protease and FN.

Figure 11 shows FN immunoreactivity in a heparin-binding, 30 kD fraction obtained from serum-supplemented SC CM.

Figure 12 shows that proteolysis of FN by plasmin generates a cryptic antiproliferative activity.

Figure 13 shows the results of electrophoresis of chromatographically isolated proteolytic FN fragments generated by plasmin.

Figure 14 compares NAP and CAFF activities for SC treated with various mitogens and for Schwann cell lines.

Detailed Description of the Invention

We have recently developed in vitro evidence that Schwann cells themselves produce distinct and very potent proteins which reversibly cause inhibition of their own proliferation in response to specific mitogens. The existence of such inhibitory activities could explain why Schwann cells do not proliferate rapidly in vitro.

The identification and isolation of the above-described antiproliferative activity requires the generation of purified rat sciatic nerve Schwann cell cultures. Culture conditions were established that would allow the maintenance of quiescent (non-dividing) Schwann cells (Example 1). Schwann cells proliferate in response to a variety of different agents such as ganglionic neurites or their fragments and agents that raise intracellular cyclic AMP levels.

The identification of the antiproliferative activity was facilitated by the use of a quantitative DNA-labelling method whereby a single microculture can be examined for: (1) cell morphology; (2) cell number; (3) DNA synthesis (with the same sensitivity as that given by the earlier radiothymidine technique); and (4) expression of cell antigens. The method involves first measuring by enzyme-linked immunosorbent assay (ELISA) of the fixed monolayer the total bromodeoxyuridine (BrdU) incorporation into DNA per culture, using a soluble chromogen (o-phenylenediamine). After BrdU-ELISA measurement, the cultures are immunostained for BrdU-positive nuclei using an insoluble chromogen (diaminobenzidine), as well as for selected cytoplasmic antigens (Example 2).

Cultured rat sciatic nerve Schwann cells hardly proliferate in 10% calf serum as determined by BrdU-ELISA. This behavior is unexpected since Schwann cell conditioned medium contains several autocrine mitogens including laminin

(a Schwann cell product) and fibronectin (a serum component) as well as TGF-ß (Ridley et al., 1989) and Glial Maturation Factor (Bosch, et al., J. Neurosci., 9, 3690-3698 (1989)). However, mitogen-stimulated Schwann cells undergo a rapid burst of proliferation immediately following culture medium changes (See Figure 1 and Example 3). In addition, non-mitogen-stimulated Schwann cells increase their proliferation slightly if the culture medium is changed frequently (e.g. every 12 hours). The production of an autocrine proliferation inhibitor by Schwann cells is demonstrated by the inhibition of mitogen-stimulated Schwann cell proliferation by conditioned medium (CM) from dense, but subconfluent Schwann cell cultures (Example 4). Schwann cell CM can completely inhibit, in a dose-dependent and reversible fashion, the proliferation of test Schwann cells under the influence of different types of mitogens (Example 5; Figure 2).

Anti-proliferative activity can be found in medium conditioned not only by rat sciatic nerve Schwann cells, but also by serum-stimulated or serum-free rat RN22 (Pfeiffer and Wechsler, Proc. Natl. Acad. Sci. USA, 69, 2885-2889 (1972)) and D6P2T (Bansal and Pfeiffer, J. Neurochem., 49, 1902-1911 (1987)) Schwannoma cells. In order to survey the distribution of antiproliferative activity and to choose a plentiful source for its further characterization, we measured the amount of antiproliferative activity toward Schwann cells in selected cell conditioned media. Different source cultures were set up under identical conditions, that is, using the same culture vessel size, media, volume, and cell density, and culture media were conditioned for 2 days before being assayed for antiproliferative activity toward test SC microcultures. The results are shown in (Table 1 of Example 8). Antiproliferative activity was detectable in all of the Schwann cell conditioned media (primary, immortalized, and transformed cells). Serum-free RN22 Schwannoma cell CM is a preferred source of antiproliferative activity since these cells (1) produce a reasonably high amount of activity per cell; (2) are more readily available due to a higher rate of

doubling; and (3) can be cultured for an extended period in serum-free medium allowing the activity to be more highly concentrated by ultrafiltration and to be relatively low in serum contaminants.

Schwann and RN22 Schwannoma activities can be isolated by concentration of conditioned medium from cell cultures by PM10 ultrafiltration and fractionation of the retentates by CL4B chromatography as described in Example 9. The antiproliferative activity from both sources occurs in three distinct forms (as shown in Figure 2A and 2B): (1) a high molecular weight form eluting in the void volume (V_o: Mr = $\geq$1000kD): (2) an Mr = 55kD form; and (3) a low molecular weight form eluting in the total volume (V_t: Mr $\leq$ 30 kD). The active 55 kD protein can be separated from the 1000 kD protein in electrophoretically pure form by resubmitting the V_o fractions to chromatography under dissociative conditions, that is CL4B with 4M urea or SDS-PAGE (Figure 5C, 5D). These substances are designated "Neural Antiproliferative Proteins", and the two species are accordingly "55 kD NAP" and "1000 kD NAP".

The glial antiproliferative activities can be quantitated in antiproliferative units (APU) based on inhibitory titer, as in Figure 2, that is, 1 APU = dilution for which 50% inhibition is achieved. Antiproliferative effect on cell cultures is determined by the percent inhibition of Schwann cell proliferation as determined by the BrdU DNA labelling procedure using untreated but mitogen-stimulated cell cultures as controls. The assay can then be used to monitor isolation of the antiproliferative activity.

Other chromatographic approaches can be used to isolate the 55 kD antiproliferative activity. According to one procedure the high molecular weight activity from the first CL4B fractionation of CM is brought to 4 M urea and fractionated on a second CL4B column equilibrated with PBS as described in Example 11(A).

The 55 kD factor was also isolated from the high molecular weight fraction from gel filtration of the serum

free RN22 CM by SDS-PAGE and assayed for antiproliferative activity as described in Example 11(B). The active material which eluted from the preparative gel was examined by analytical SDS-PAGE, electroblotting and colloidal gold staining, and showed that this preparation of the 55 kD antiproliferative factor was electrophoretically pure.

We have therefore isolated a protein from Schwann cell conditioned medium and have evidence that it is a protein of approximately 55 kD synthesized by both Schwann cells and Schwannoma cells. The protein has a potent, but reversible, antiproliferative effect on glial cells, and can also exist in and be isolated from a high molecular weight (1000 kD) complex as well as in the 55 kD form. The activity of the protease can be abolished after heat (90°C) or pronase treatment. The protein is designated as 55 kD NAP.

The biochemical properties of the two NAP species differ in that the free 55kD activity, but not the 1000kD activity is abolished after heat (90°C) or pronase digestion, suggesting that either the two forms of NAP are different, or that, when complexed, the 55kD species is protected from heat or pronase. The free 55Kd species in not inactivated, but appears to become more active by limited proteolysis. Zymography of the purified 55kD species in SDS gels containing gelatin substrate confirms that a protease activity is associated with or copurifies with the purified 55kD NAP. Further zymography with metal chelator-treated gels (e.g. using EDTA and o-phenanthroline) suggests that the 55kD species is a calcium-dependent, Zn^{++}-containing metalloprotease.

Experimental evidence suggests that Schwann and Schwannoma cells synthesize the 55kD protease. Zymography performed on conditioned medium fractions from either Schwann or RN22 Schwannoma cells detects a comigrating 55kD metalloprotease band. On metabolic labelling of cultured Schwann or Schwannoma cells with ^{35}S-methionine and subsequent fractionation (as in Figure 2) of the labelled medium the isolated 55kD protease band is radiolabelled. Thus these

cells produce an autocrine regulator of their own proliferation.

The 55kD protease activity further has the ability to act on fibronectin to produce cryptic antiproliferative fibronectin fragments, having antiproliferative activity of their own. Antiproliferative fragments can be derived by proteolytic degradation of human, bovine and rat fibronectins. An active fibronectin fragment can be generated as well by limited proteolysis of intact fibronectin by the activity of proteases other than that associated with the 55 kD NAP.

The fibronectin fragments isolated and found to have potent antiproliferative activity for mitogen-stimulated Schwann cells are 29-30 kD proteins that bind heparin. These proteins have been designated Cryptic Antiproliferative Fibronectin Fragments (CAFFs).

This activity was originally noted during binding studies for the 55 kD metalloprotease activity. The fact that the 55kD activity also occurred in a complex suggested that other molecules could bind to it. We coated microwells with 1000kD complex from which the 55kD activity had been removed by dissociative chromatography (i.e. in the V_o pool in Figure 2C). Other microwells were coated with potential components of the V_o fraction such as laminin, collagen Types I or Type IV, or fibronectin. A known amount of the purified 55kD protein was then incubated in each coated well and the unbound material assayed for residual antiproliferative activity. Only about 20% of the activity was recovered from 1000 kD complex-coated wells, suggesting that the 1000 kD complex retains binding capacity for the 55 kD NAP. Full NAP activity was recovered from laminin and collagen-coated wells, suggesting that the 55 kD species was not sequestered by these extracellular matrix proteins. However, significantly more antiproliferative activity was recovered from fibronectin-treated wells than was originally added. The unexpected result suggested that the 55 kD metalloprotease activity may generate active fibronectin fragments. We then incubated the 55 kD NAP, or even the crude conditioned media from Schwann or

RN22 cells, for 48 hours at 37°C with intact fibronectin and submitted the digest to SDS-PAGE. Coomassie Blue staining showed that very little intact 440 kD fibronectin remained but a variety of different sized fragments resulted (Mr = 20-160 kD). When the fibronectin digest was applied to successive gelatin-sepharose, DEAE cellulose, and heparin-sepharose chromatography columns, a fibronectin proliferative activity was recovered only from heparin (Example 11). Control experiments showed that the purified 55 kD NAP species by itself does not bind to gelatin, DEAE or heparin and thus was separated from the fibronectin fragments during heparin-sepharose chromatography. The fibronectin-derived heparin-binding antiproliferative activity was isolated further by gel filtration and the active fraction exhibited only one 30 kD band by SDS-PAGE. This is the substance that is called CAFF. Thus, this fibronectin fragment (CAFF) does not possess the gelatin binding region and probably does not contain the fibronectin cell binding domain, RGD.

In experiments for which the results are presented in Figures 8-14, support is provided for the immunological relationship between stromelysin and the 55 kD NAP; the production of a stromelysin-like protein in SC CM; and the antiproliferative activity of the N-terminal fragment of fibronectin.

In one experiment, a highly enriched preparation of the 55 kD NAP obtained from serum-free RN22 CM was examined by non-reducing SDS-PAGE and protein staining (Figure 8A: lane 1). On zymographic gels, the 55 kD NAP contained a caseinolytic activity (lane 2) which was eliminated when zymography was performed in the presence of the zinc chelator 1,10 phenanthroline (lane 3). The RN22 CM-derived NAP was examined for anti-stromelysin immunoreactivity by western immunoblotting (lane 4). Molecular weight designations represent the migration positions of BSA (67 kD) and ovalbumin (46 kD) on 12% acrylamide standard and zymographic gels and on western blots. Figure 8A.

Crude serum-free CM was collected after 2 days from dense cultures of RN22 Schwannoma (Figure 8:lane 1) and isolated SCs (lane 2) and then concentrated 300-fold by ultrafiltration (10 kD cutoff). The concentrate (20μl) was run on 12% acrylamide minigels under non-reducing conditions, electroblotted to nitrocellulose, and immunoperoxidase-stained using monoclonal antistromelysin antibodies as described. Molecular weight designations represent the migration positions of BSA (67 kD), ovalbumin (46 kD), and carbonic anhydrase (30 kD). Figure 8B.

The SC-derived 55 kD metalloprotease generated proteolytic FN fragments. (Figure 9) Bovine plasma FN (1 mg) was mixed with about 8 mg of the 55 kD metalloprotease and incubated for 72 h at 37°C in 25 mM Tris-HCl, pH 7.6, containing NaCl (100 mM), $CaCl_2$ (5 mM), APMA (1 mM), PMSF (0.5 mM), NEM (10 mM), and aprotinin (1μg/ml). Incubation was performed in the presence (lane 1) or absence (lane 2) of the metalloprotease inhibitor, 1,10-phenanthroline. The samples were electrophoresed on 5-15% acrylamide gradient gels under non-reducing conditions and then stained with Coomassie blue. The digest shown in lane 2 was applied to tandem columns of DEAE-, gelatin-, and heparin-Sepharose. The fragments which did not bind to DEAE or gelatin but did bind to heparin were eluted from the heparin column by 0.5 M NaCl. The heparin-binding, 0.5 M NaCl-eluted fraction was concentrated by ultrafiltration (10 kD cutoff) and was further fractionated by S200 (superfine) gel filtration in PBS. The fractions corresponding to a molecular mass of about 30 kD were pooled and examined by SDS-PAGE (lane 3) as described above. The migration positions of myosin (200 kD), phosphorylase B (92 kD), and carbonic anhydrase (30 kD) are indicated.

A mixture of the 55 kD protease as described in the experiment above and FN has antiproliferative activity (Figure 10). Bovine FN was degraded by incubation with the 55 kD protease as described for Figure 9, lane 2. The proteolytic mixture was concentrated and fractionated by gel filtration and the eluted fractions were assayed for antiproliferative

activity using cholera toxin-stimulated SCs as described. Estimated molecular masses for the two peaks of antiproliferative activities are indicated by arrows. The data of Figure 10 represent the means of duplicate determinations from two separate experiments.

The 30 kD fraction obtained from serum-supplemented SC CM has FN immunoreactivity (Figure 11). Serum-supplemented medium conditioned for 3 days by quiescent SC cultures was applied to tandem columns of DEAE-, gelatin-, and heparin-sepharose. The material which did not bind to DEAE or gelatim but did bind to heparin was eluted from the heparin column by 0.5 M NaCl and then was submitted to gel filtration. The fractions corresponding to a molecular mass of about 30 kD were pooled and examined by SDS-PAGE and protein staining (lane 1) and western immunoblotting using polyclonal anti-FN antibodies (lane 2). The migration positions of carbonic anhydrase (30 kD) and soybean trypsin inhibitor (21 kD) are indicated.

Plasmin also generates a cryptic antiproliferative activity by proteolysis of FN. Figure 12. Bovine FN and BSA were degraded with plasmin as described, and the proteolytic mixtures were tested for antiproliferative activity using cholera toxin-stimulated SCs. Intact FN was additive with the mitogenic effects of cholera toxin. The mixture of FN and plasmin, after 16 h of incubation, expressed a potent antiproliferative activity capable of completely inhibiting DNA synthesis. Plasmin, within the concentration range used to degrade FN, did not inhibit SC proliferation and the proteolytic mixture of plasmin and BSA expressed no antiproliferative activity. Data of Figure 12 represent the means of quadruplicate determinations from two separate experiments. In a related experiment, reported in Figure 13, the molecular weight of the plasmin generated FN fragment was determined on electrophoretic gels. Bovine plasma FN (25 mg) was digested with 500 μg of porcine plasmin for 20 h at 37°C in 25 mM Tris-HCl, pH 8.4, containing NaCl (100 mM) and lysine

(10 mM). The digest (lane 1), was applied to tandem columns of DEAE-, gelatin-, and heparin-sepharose. The DEAE and gelatin non-binding FN fragments that bound to heparin were eluted from the heparin column with 0.5 M NaCl. The eluted fraction was concentrated by PM10 ultrafiltration and them was submitted to S200 (superfine) gel filtration. The fraction corresponding to a molcular mass of about 30 kD was examined by SDS-PAGE immediately (lane 2) or after being stored in solution for 7 days at 4°C (lane 3). Samples were run under non-reducing conditions on 5-15% acrylamide gels which were then stained with Coomassie blue. The migration positions of myosin (200 kD), phosphorylase b (92 kD), and carbonic anhydrase (30 kD) are indicated.

In yet another experiment, the activity of NAP and CAFF on mitogen-stimulated SC and on Schwann cell lines was determined. Figure 14. Subconfluent microcultures of rat sciatic nerve SCs (14,000 cells/6 mm diameter wells) were grown with 100 μl DMEM containing 10% calf serum with one of the following mitogens: soluble cholera toxin (20 ng/ml); polyornithine-treated wells coated with rat laminin (50 μl/well, 2 μg/ml); or co-culture with ciliary ganglionic neurons (2000 neurons/well). These mitogen-stimulated SCs were treated for 72 h with serial dilutions of the (A) 55 kD NAP or (B)CAFF (29 kD, amino-terminal FN fragment generated by the 55 kD protease). Immortalized SC and two schwannoma cell (RN22 and D6P2T lines) cultures were grown in DMEM containing 10% calf serum and treated with the (C) 55 kD NAP or (D) CAFF as described above. Proliferation was assessed by addition of BrdU (10 μM) to the media during the final 24 h of the 72 h treatments and immunoassays of BrdU incorporation nto DNA were performed as described. Values for each condition were expressed as the percentage of maximal [BrdU]DNA immunoactivity for cultures without NAP or CAFF treatment. Data of Figure 14 represent the means of quadruplicate determinations from four separate experiments.

The evidence for a antiproliferative protease synthesized within Schwann cells and its action on fibronectin is the basis for a proposed model for the autocrine regulation of Schwann cell proliferation which is depicted in Figure 7.

Utility

The antiproliferative properties of the NAP and CAFF proteins herein described have the ability to inhibit the proliferation of normal glial cells (Schwann cells, oligodendroglial cells or astroglial cells) and glial tumor cells (Schwannoma and glioblastoma). Accordingly they are useful in promoting neural regeneration following trauma or surgery or in inhibiting the growth of certain tumors of nervous tissue.

Brain or spinal cord tissue subjected to physical damage (wounds) or diseases often responds by gliosis, whereby astroglial cells proliferate to form scarring lesions through which regeneration of new neural tissue is blocked or inhibited. The gliosis reaction usually peaks at one week post injury. We have found that mitogen-stimulated astroglial cell proliferation in vitro is inhibited by NAP and CAFF. Thus, administration of antiproliferative factor in this period may act to inhibit astroglial growth to allow the regeneration of healthy functional nerve tissue.

Similarly, antiproliferative factor may be used to inhibit the growth of tumors of the nervous tissue. The antiproliferative factors, or NAPs or CAFFs of the invention, are shown to inhibit the growth of many types of glial nerve cells, including Schwann, oligodendrocytes, and astrocytes, although not meningeal or fibroblast cells. Growth of gliomas, or tumors arising from these cells, may also be effectively inhibited by their administration.

Either the 55 kD NAP protein or the CAFF protein may be used for these purposes, and they may be used in either partially purified or pure form. Local administration of the substances is preferred, and most preferred is administration in a solid, biodegradable matrix, which has the advantage of maintaining an effective dose of the antiproliferative agent

at the affected site for a period of time, for example, several days or weeks.

Since cell cultures of neurons used in the isolation and evaluation of the antiproliferative factors disclosed herein may be susceptible to the same inhibitory responses as the same cells in vivo, it is anticipated that an effective dose of the antiproliferative activities may be similar to those that inhibit in vitro cell growth, although a greater concentration of dose should be administered to allow for tissue dilution. Accordingly, effective concentrations of NAPs for the purposes proposed can be in the range of as low as picomolar concentrations, which have been shown to produce detectable inhibition of glial cell growth, and up to doses in the micromolar or millimolar concentration, which produce total inhibition with no apparent toxicity. Preferably, dose concentrations are in the picomolar to millimolar concentration, and most preferably in the range of 10 picomolar to 1 millimolar concentration. Duration of treatment may be transient or for a period of several days, weeks or even months as required to produce the desired inhibition of glial cell growth.

Longer term administration of the glial antiproliferative agents is preferably in the form of the slow release biodegradable implant devices which act to maintain a stable effective concentration of agent at the site of the lesion. Any of the known bioerodable, biodegradable polymers may be used in the present invention. These include polymers of glycoside, lactic acid, lactide, glutamic acid, collagen, and albumin, as well as other known materials. Polylactic and copolymers of lactic acid are particularly preferred. Lactic acid is present in living tissue, including brain tissue, as a product of glycolysis. The polymer of lactic acid does not invoke an immunological response in man, and is commonly used as a biodegradable suture material. The polylactic material used in the invention may be a low molecular weight polymer having the consistency of paste or putty. Such polymers of lactic acid have molecular weights of less than 5000,

preferably from about 800 to 1000. Compositions may be prepared by the simple mixing of the polymer with the antiproliferative NAP proteins or the fibronectin fragments described.

The controlled release polymer matrix, comprising a biocompatible, bioerodable polymer and an inhibitor of glial cell proliferation may be implanted within the body of an animal at the site of injured nervous system tissue. The matrix may be implanted, for example, intracranially in a human afflicted with a glioma, the most common form of brain tumor. The procedure may first involve the removal of any localized solid glial tumor, for example, a glioblastoma, from the brain prior to implanting the bioerodable polymer matrix, and then implanting the bioerodable polymer matrix into the site from which the malignant tissue is removed. The polymer, in soft malleable form, may be shaped to fit the site from which the tissue was removed such that it opposes or effaces the residual tumor bed.

The NAP antiproliferative proteins of the invention or alternatively the glial cell inhibiting fragments generated by the 55 kD NAP from fibronectin may also be introduced to the site of nervous tissue injury or tumor growth in the central nervous system by means of a cannula inserted into a ventricle of the brain.

The antiproliferative agents of the invention may be administered similarly, that is locally in the form of various pharmaceutically suitable vehicles, including controlled release bioerodable materials, to nervous tissue lesions of the peripheral nervous system as well as those of the central nervous system. One example of advantageous use is in the therapy of neurofibromatosis, or Von Recklinghausen's disease, wherein Schwann cells of the neurilemmal sheath proliferate in abnormal masses along nerves to produce multiple pedunculated soft tumors (neurofibromas) under the skin of the entire body.

Suitable pharmaceutical preparations may comprise aqueous or oily fluids, pastes, gels, ointments or powders, that are biocompatible. They may further comprise encapsulation of the

active agent in porous chambers, such as for example, those prepared from natural or artificial membranes.

B. NAP proteins and the therapy of demyelinating disease.

According to another aspect of the invention, in view of the metabolic role of the 55 kD protease and the 30 kD fibronectin fragment appear to play in the regulation of glial cell proliferation, agents that oppose the activity of either protein can be usefully applied in the therapy of diseases or syndromes characterized by demyelination.

Demyelinating diseases, comprising acute disseminated encephalomyelitis, (including postinfectious and postvaccinial encephalomyelitis), acute necrotizing hemorrhagic leukoencephalitis, multiple sclerosis, and diffuse cerebral sclerosis share the common pathologic feature of foci of degeneration, involving the myelin sheath of nerves. The destruction of myelin is considered the primary change (Poskanser, D. and Adams, R., Harrison's Principles of Internal Medicine, McGraw-Hill, New York, 1970, Chap.362). Multiple sclerosis is one of the most common chronic neurological diseases. It is characterized by demyelinated lesions of the spinal cord having a pinkish gray appearance (due to the loss of myelin) which stand out to the surrounding white matter. Myelin is laid down around tissues of the peripheral nervous system by the Schwann cells of the nerve sheath, and it has been observed that noxious agents that attack Schwann cells cause demyelination of peripheral nerve. Remyelination can restore the function of denuded segments of an intact axon. Therefore agents which facilitate or accelerate Schwann cell proliferation could achieve remyelination. Application of anti-NAP agents, either antibodies to NAPs or 30 kD fibronectin fragments (or synthetic peptides corresponding to sequences within these two substances), chemicals such as protease inhibitors or fibronectin peptide antagonists could be therapeutically useful in opposing the regulating antiproliferative effects of NAPs to promote proliferation in lesions where glial cell growth is limiting, that is, either demyelinating diseases or

traumatic injury. Antibody reagents can stimulate glial cell proliferation which would normally be suppressed by endogenous NAP. Demyelinating disease such as multiple sclerosis are characterized by low numbers of oligodendroglial cells in brain and cord, and NAP-inactivating agents, such as antibodies could be usefully administered to stimulate the local production of oligodendroglia to the extent that there will be enough to remyelinate the hypomyelinated areas.

Antibodies to NAPs such as, for example, the 55 kD protease or the 30 kD fibronectin fragment disclosed herein may be produced according to known techniques of immunizing animals, such as rabbits or goat and collecting the antibodies in a serum sample when immunity has been achieved as determined, for example, by the ability of the antibodies in the serum to bind the labelled antigen in a binding assay. Monoclonal antibodies to NAPs can be produced by any procedure known to those skilled in the art, for example, based on the fundamental procedure of Kohler, and Milstein, Nature 256(8):295-297 (1978). Briefly, according to this technique, animal lymphocytes are stimulated (immunized) either in vitro or in vivo by a preparation of antigen, in this case, any NAP agent. An animal may be injected either intraperitoneally or intravenously or both. The dose of antigen may be between approximately 1μg and 50 μg/mouse, preferably between approximately 5μg and 10 μg/mouse. A second dose of antigen is administered at least 3 weeks, and preferably 5 weeks after the first immunization.

The immunized lymphocytes are fused with myeloma cells that are sensitive to hypoxanthine-aminopterin-thymidine (HAT) medium, to allow selection of hybrids to be accomplished by growth in HAT medium. Several mouse or human myeloma lines suitable for the fusion are available from the ATCC cell line collection (Rockville, Maryland). Fusion is mediated by polyethylene glycol. Hybrids are placed in HAT medium in microtiter wells two days after fusion, and the culture media is screened for monoclonal activity by binding assays,

preferably enzyme-linked immunoassay using bound immunizing antigen.

The antibodies produced would be tested for their ability to prevent the antiproliferative activity on cultured glial cells. If the antibody stopped the antiproliferative activity, the antibody would be detected because it acts like a mitogen. Such antibodies would be used to stimulate proliferation of glial cells in vivo where there may be a need to increase glial number. For example, in multiple sclerosis, the number of oligodendroglial cells may be 1/10th that of normal brain. Patients having demyelinating disease may be treated with the antibodies to NAP agents according to the protocol recited herein for the NAP agents themselves in the treatment of glial cell tumors or nervous tissue trauma.

The present invention is described below in detail using the following examples, but the methods described are broadly applicable for the preparation of all of the substances described herein and are not limited to the examples given below.

EXAMPLE 1

Cell Culture

Purified populations of quiescent SCs were obtained from dissociated neonatal rat sciatic nerves as previously described (Muir, et al., J. Cell. Biol., 109, 2353-2362 (1989a)). To generate large numbers of cells, the primary Schwann cells were expanded in the presence of cholera toxin as mitogen. Dense (but subconfluent) SC cultures (3-4 X 10^6 cells/100 mm dish) were grown in Dulbecco's modification of Eagle's medium supplemented with penicillin and L-glutamine (1 mM) (DMEM) containing 2 or 10% calf serum, and CM was collected every two days. Loss of normal growth control (immortalization) of secondary SCs was achieved by continuous treatment with 20 ng/ml cholera toxin (Sigma, St. Louis, MO.) and passaging 1:6 every 4 days for 2-3 months. The immortalized SCs were further passaged 1:10 every 5 days for 1-2 months without cholera toxin. RN22 (Pfeiffer and

Wechsler, Proc. Natl. Acad. Sci. USA, 69, 2885-2889 (1972)) and D6P2T Schwannoma cells (Bansal and Pfeiffer, J. Neurochem., 49, 1902-1911 (1987)) were cultured in DMEM containing 10% calf serum. For the collection of serum-free RN22 CM, dense (3-4 X 10^6 cells/100 mm dish) cultures were rinsed twice with phosphate-buffered saline (PBS) to remove serum proteins and the cells were grown in serum-free DMEM for 2 days before medium collection. Serum-containing and serum-free CM were also collected from rat brain astroglia (prepared according to Rudge, 1986), oligodendroglia (McCarthy, K.D. and de Vellis, J., J. Cell Biol. 85:890-902 (1980)), sciatic nerve and mouse 3T3 fibroblasts. Cells were determined to be free of mycoplasma.

EXAMPLE 2

Schwann Cell Proliferation Assay and BrdU DNA Immunostaining.

Quiescent SC microcultures were established in polyornithine treated 96-well plates containing 14,000 cells/6 mm well in 100 µl of DMEM+10% calf serum as described (Muir, et al., J. Cell. Biol., 109, 2353-2362 (1989a)). For some assays, SCs were seeded in polyornithine-coated wells treated with 50 µl of a 2 µg/ml solution of rat L2 yolk sac tumor laminin (prepared as described by Engvall, E. et al., Arch. Biochem. Biophys. 222: 649-656 (1983)). SC/neuron co-cultures were established adding 14,000 SCs and 2,000 embryonic day 8 ciliary ganglionic motor neurons to each microwell as described (Muir, et al., J. Cell. Biol., 109, 2353-2362 (1989a)). For routine assays of antiproliferative activity on mitogen-stimulated SCs, microcultures were seeded in DMEM+10% calf serum containing 20 ng/ml cholera toxin. Serially diluted test samples were presented for 72 h and bromodeoxyuridine (BrdU) (Sigma) was added to a concentration of 1 µM during the final 24 h. SC proliferation was assessed by direct cell counting and BrdU incorporation into DNA was measured by an enzyme-linked immunosorbent assay performed on

fixed monolayer microcultures as previously described (Muir Varon, and Manthorpe, Anal. Biochem. 185:377-382 (1990b)). Briefly, following BrdU incorporation, the cells were fixed by 70% ethanol and the DNA denatured by incubation with 2 M HCl for 10 min at 37°C. BrdU-DNA was labeled using monoclonal anti-BrdU antibody (Dako-Patts Corp., Santa Barbara, CA.) (50 µl/well; 1 µg/ml) and bound antibody was detected by peroxidase-conjugated rabbit anti-mouse IgG (Dako-Patts) (50 µl/well; 2 µg/ml). The colorimetric substrates o-phenylenediamine (0.05%) and H_2O_2 (0.02%) were added in 50 mM phosphate/citrate buffer at pH 5 and the reaction was terminated after 5-20 min by the addition of sulfuric acid. The absorbance was measured at 490 nm by a MR600 Microplate Reader (Dynatech Labs, Alexandria, VA.) interfaced with a computer. Using cholera toxin-stimulated SCs, the titer of each sample in antiproliferative units (APU)/ml was expressed as the sample dilution required to inhibit by 50% the maximal incorporation of BrdU into DNA (BrdU-DNA immunoactivity). The percentage of cells with BrdU-DNA was determined by immunostaining essentially as described above in the ELISA except the insoluble chromogen diaminobenzedine-tetrahydrochloride was used. Proliferation assays using immortalized SC and rat schwannoma cell lines were performed as described above for SCs except that no mitogens were added to the serum-supplemented medium.

EXAMPLE 3

Schwann Cell Proliferation in Response to Cholera Toxin and Medium Changes

Figure 1. SC cultures (20,000 cells/cm^2) were treated for 3 d with 20 ng/ml cholera toxin in DMEM containing 10% calf serum without changing the medium and then the medium was removed and the cultures were grown for the next 3 d in 1 part DMEM containing 10% calf serum and 3 parts phosphate buffered saline. Cell proliferation was examined in replicate cultures by the addition of BrdU (1µM) to the culture medium for 24-h

periods throughout the 6 d experiment. After each 24-h period the assay was terminated by ethanol fixation and the cells were immunostained for BrdU incorporation into DNA as described in Materials and Methods. Data represent the mean of four determinations (50 cells each) per dish and each condition was duplicated in each of two separate experiments. (Std. Dev. < 8%).

EXAMPLE 4

Schwann Cell Response to Mitogens and Inhibition by Schwann cell CM.

Figure 2. Subconfluent microcultures of rat sciatic nerve SCs (14,000 cells/6 mm diameter well) were grown with 100 μl DMEM containing 10% calf serum with one of the following mitogens: soluble cholera toxin (20 ng/ml); polyornithine-treated wells coated with rat laminin (50 μl/well, 2 μg/ml); or co-culture with ciliary ganglionic neurons (2000 neurons/well). These mitogen-stimulated SCs were treated with serial dilutions of CM from dense SC cultures for 72 h. SC proliferation was assessed by addition of BrdU (1 μM) to the media during the final 24 h and (A) immunoassay of BrdU incorporation into DNA and (B, C) immunostaining of BrdU-labeled nuclei were performed as described in Example 2. Values for each condition in (A) were expressed as the percentage of mitogen-stimulated [BrdU]DNA immunoactivity for the corresponding mitogen without SC CM treatment. For cholera toxin-stimulated cultures (B) and cholera toxin-stimulated cultures treated with 50% SC CM (C) BrdU-labeled nuclei were immunostained using diaminobenzedine-tetrahydrochloride and unlabeled nuclei were lightly counterstained with toluidine blue. Data represent the means of quadruplicate determinations from four separate experiments. Cholera toxin and laminin, Std. Dev. < 6%; Neurons, Std. Dev. < 16%.

EXAMPLE 5

Reversibility of the Antiproliferative Activity

Figure 3. SC microcultures were treated with (A) cholera toxin, (B) laminin, or (C) ciliary neurons as described in Figure 2. BrdU incorporation in response to each mitogen was examined over a 4 or 5 d period (solid circles). A second set of mitogen-stimulated SC cultures were treated with SC CM (a 1/10 dilution of SC CM that was concentrated 10-fold and dialyzed) from the onset of the assays (closed triangles). A third set of replicate cultures was treated with SC CM (as above) from the onset of the assays except that at 2 d the treatment medium was removed and replaced with fresh, unconditioned medium (open triangles). BrdU was added during the final 24 h of each test interval (days 0-1, 0-2, 0-3, 0-4, and for neurons 0-5) and the assays were terminated by ethanol fixation. [BrdU]DNA was measured by ELISA and immunoactivity was expressed as a percent of the maximum value obtained for each mitogen. Data represent the means of two experiments each having quadruplicate determinations. A and B, Std. Dev. < 8%; C, Std. Dev. < 14%.

EXAMPLE 6

Responsiveness of Transformed SC Types to the Antiproliferative Activity.

SCs treated with cholera toxin (20/ng/ml), immortalized SCs, and RN22 and D6P2T Schwannoma cells were cultured in microwells (14,000 cells/well) and treated for 72 h with serial dilutions of serum-free RN22 CM (concentrated 300 fold). BrdU was added to the microcultures for the final 24 h and [BrdU]DNA was measured by ELISA (Figure 4). Data represent immunoactivity expressed as a percent of the maximum value obtained for each actively proliferating cell type. Std. Dev. < 4%.

EXAMPLE 7

Metabolic Labeling with [^{35}S] Methionine

Protein synthesis by SCs in vitro was quantified by the incorporation of radiolabeled amino acids. Quiescent SCs were cultured in DMEM (98% methionine-free DMEM + 2% DMEM) containing 10% dialyzed calf serum and 10 μCi/ml Tran^{35}S-label (ICN Biochemicals Inc., Irvine, CA.) in the absence and presence of CM or fractions containing antiproliferative activity. After 72 h the cultures were washed several times with Hank's balanced salt solution, hydrolyzed with 0.2 N NaOH at 37°C for 30 min, and then mixed with Liquescent (National Diagnostics, Manville, NJ.) and counted.

EXAMPLE 8

Antiproliferative Activity toward Schwann Cells: Comparison of Conditioned Media from Different Sources

Cells in logarithmic growth were detached and dissociated by trypsin/EDTA and then 2 X 10^6 cells were seeded in 75 cm^2 tissue culture dishes. The cultures were grown in DMEM supplemented with 10% calf serum (or DMEM only for serum-free RN22). The cultures were grown for 48 h and then the cells were counted and the CM collected. SC + CTx cells were treated with 20 ng/ml of cholera toxin for two days prior to and 24 h after seeding in the 100 mm dishes. The effects of cholera toxin on SC proliferation persists for at least two days following its removal from SC cultures. During the 48-h CM collection period, SC + CTx medium did not contain cholera toxin although a high rate of cell division continued. The CMs were assayed for the ability to inhibit incorporation of BrdU into DNA by mitogen-stimulated SCs as described. The titer of each CM was expressed as antiproliferative units per ml of CM (APU/ml) and normalized to an average cell number (the mean of initial an ending cell numbers) present during the 48 h CM collection period. Data in Table 1 represent the means of 8 determinations from 4 separate CM collection experiments (S.D. < 10%).

Table I. Antiproliferative activity toward Schwann cells: Comparison of conditioned media from different sources.

Source	mitotic cycle (d)	APU/ml/10^6 cells
SC	16	5.1
SC + CTx	2-3	1.9
Immortal SC	2	1.2
RN22 (+serum)	0.8	6.8
RN22 (serum-free)	1-2	2.6
D6P2T	0.7	7.3
Astroglia	ND	<0.1
Oligodendroglia	ND	<0.1
Meningeal cells	ND	<0.1
Fibroblasts (nerve)	ND	<0.1
3T3 fibroblasts	ND	<0.1

EXAMPLE 9

Isolation of Antiproliferative Activity from Culture Medium

A. Concentration of conditioned media.

Medium conditioned by SCs or RN22 Schwannoma cells was filtered (0.2 μm pore). The CM was concentrated by ultrafiltration using a 10 kD cut-off filter (YM10, Amicon, Danvers, MA.) and then dialyzed with PBS. 150 ml of SC CM was concentrated 30-fold by ultrafiltration (10 kD cut-off) dialyzed with PBS, and then submitted to a CL4B gel filtration column in the same buffer. Similarly, 1500 ml of serum-free RN22 CM was concentrated 300-fold by ultrafiltration, dialyzed and analyzed by CL4B gel filtration. The elution profiles are shown in Figure 5A and 5B.

B. CL4B Chromatographic fractionation

The CM concentrates were fractionated by CL4B (Pharmacia, Piscataway, NJ.) gel filtration (2.5 X 96 cm column) in PBS. Eight ml fractions were collected and assayed for antiproliferative activity. The titers were expressed in

antiproliferative units/ml as described above. All titrations were performed in duplicate and four determinations were made from two separate fractionations. A and B, S.D. < 5%. Several molecular weight markers were used and the elution of ovalbumin (45 kD) is shown (Figure 5). Vo indicates the high molecular weight (> 1,000 kD) void volume and Vt indicates the total column volume. The antiproliferative activity from both sources occurs in three distinct forms: (1) a high molecular weight form eluting in the void volume (V_o: Mr = ≥1000kD): (2) an Mr = 55kD form; and (3) a low molecular weight form eluting in the total volume (V_t: Mr ≤ 30kD).

In the isolation of NAP's from Schwann cell conditioned media (SC CM, Figure 5) containing an initial 1500 APU (10 APU/mg; 5 ml of 30X concentrated CM), about 25% if the recovered activity eluted in the high molecular weight void volume, 50% in the 45-55 kD fractions, 20% in the total volume fractions, and 5% of the activity was unaccounted for). The highest specific activity of 28 APU/mg total protein was found in fractions eluting in about 420 ml, corresponding to a molecular mass between 45-55 kD (Figure 5A).

Starting serum free RN22 CM contained about half of the antiproliferative activity as Schwann cell CM but over 50 times the specific activity (380 APU/mg). For a sample containing 7500 APU (380 APU/mg; 5 ml of 300X concentrated CM), 50% of the recovered activity eluted in the high molecular mass void volume (Figure 5B), 30% in the fractions corresponding to 45-55 kD, 5% was in the total volume fractions, and 20% of the activity was unaccounted for. The highest specific activity of 800 APU/mg protein was found in fractions eluting in about 420 ml which corresponded to a molecular mass between 45-55 kD. (Figure 5B). The fractions were assayed for antiproliferative activity on SCs as described above. Molecular weight estimation was performed using an S200 (superfine, Pharmacia) gel filtration column (1.0 x 150 cm) equilibrated with PBS.

EXAMPLE 10

Isolation of the 55 kD Antiproliferative Factor

A. Isolation of 55 kD NAP from 1000 kD protein under dissociative conditions using urea.

Figure 5C. Dissociation of the 55 kD antiproliferative factor from the high molecular weight complexed form. The high molecular weight void volume fractions from CL4B gel filtration of serum-free RN22 CM (180-204 ml; 3000APU total, 200 APU/mg),(cf. Figure 5B) were pooled, concentrated and then dialyzed with 4 M urea in PBS. The dissociated sample was submitted a second time to CL4B gel filtration and was eluted with PBS. Eight ml fractions were collected and assayed for antiproliferative activity. Fractions containing urea, which eluted in the column Vt, were dialyzed with PBS before they were assayed. The results are shown in Figure 7. The 55 kD fraction obtained has a specific activity of 300,000 APU/mg protein, corresponding to a 800-fold purification. The purified factor elicited 50% inhibition of SC proliferation at a concentration of 65 pM.

C. Purification of the 55 kD antiproliferative factor by SDS-PAGE.

Figure 5D. SDS-PAGE was carried out according to the procedure of Laemmli (1970), using 12% acrylamide gels under non-reducing conditions. CM-derived active fractions were electrophoresed at 4°C, then the gel was washed thoroughly with 2.5% Triton X-100 to remove SDS and with PBS to remove the Triton. The gel was then cut horizontally into 2.5 mm slices and the slices minced. The proteins were eluted by diffusion into PBS, the pieces were removed for centrifugation, the supernatants were dialyzed with PBS and then assayed for antiproliferative activity. The active samples were rerun on SDS-gels and the electroblotted to nitrocellulose sheets according to the methods of Towbin, et al. (1979). The sheets were stained for protein using colloidal gold (Aurodye forte, Janssen, Piscataway, NJ.).

The low molecular weight void volume fractions (180-204 ml) from CL4B gel filtration of serum-free RN22 CM (cf. Figure 5C) were pooled and concentrated. The sample was dissociated with SDS and electrophoresed under non-reducing conditions on a preparative 8% acrylamide gel. The gel was washed to remove SDS and then cut horizontally into 2.5 mm slices. The proteins were eluted from the slices and then assayed for antiproliferative activity. The active fractions were rerun on analytic SDS-gels and then electroblotted to nitrocellulose sheets which were stained for protein using colloidal gold. Molecular weight markers were albumin (67 kD) and ovalbumin (45 kD).

EXAMPLE 11

Isolation of an Antiproliferative Factor from Fibronectin

Intact fibronectin was incubated with either 55 kD NAP or with plasmin and both digests were passed over successive gelatin-sepharose (to remove intact fibronectin and gelatin-binding fragments); DEAE (to remove plasmin and other high molecular weight fragments) and heparin-sepharose (to remove the non-binding 55 kD protease). The heparin column was eluted with salt, dialyzed and submitted to gel filtration. The resulting 29-30 kD fragments presented to mitogen-stimulated rat nerve Schwann cells. Fragments from fibronectin digested with the 55 kD NAP or plasmin inhibit Schwann cell proliferation with similar $ED_{50's}$ corresponding to about 100 ng/ml (Figure 6).

EXAMPLE 12

Antiproliferative Activity in Fractions of Proteolytic Fibronectin Fragments

Three preparations containing FN fragments were found to express potent antiproliferative activity for SC's. The proteolytic samples were prepared as follows:

(55 kd protease + FN)

1 mg of purified FN was mixed with about 8 μg of SC-derived, APMA-activated, 55 kD metalloprotease preparation and incubated for 72 h at 37·C in 25 mM Tris-HCl, pH 7.6 containing NaCl (100 mM), $CaCl_2$ (10 mM), PMSF (0.5 mM), NEM (10 mM) and aprotinin (1μg/ml);

Plasmin + FN

25 mg of FN was digested with 500 μg of porcine plasmin for 20 h at 37°C in 25 mM Tris-HCl, pH 8.4 containing NaCl (100 mM) and lysine (10 mM);

30 kD SC CM

Serum (containing FN) supplemented medium was added to pure SC cultures for 72 h. The SC CM was concentrated and a 30 kD fraction was obtained by gel filtration. Each sample was adjusted to pH 8.0 and then was applied to columns of DEAE-, gelatin-, heparin-, and actin-sepharose. The bound material were eluted and all factions were assayed for antiproliferative activity using cholera toxin-stimulated SCs (scored as expressing [+] or not expressing [-] activity. IN addition, the samples were submitted to affinity-chromatographies in sequence and a final heparin-binding antiproliferative fraction was further purified by S200 gel filtration. A major peak of antiproliferative activity was eluted in fractions corresponding to about 30 kD which was then examined by SDS-PAGE. Purified fragment preparations were submitted to dansylation and direct Edman degradation for amino-terminal analysis.

	Antiproliferative Activity in Sample attributable to the FN fragment*		
Property	55 kD protease + FN	Plasmin + FN	30kD SC CM
DEAE binding	-	-	-
Gelatin-binding	-	-	-
Heparin-binding	+	+	+
Actin-binding	+	+	n.d.
N-blocked	+	+	n.d.
Molecular Mass	29 kD	29 kD	29 kD

*The proteolytic samples inherently contained antiproliferative constituents with known properties; the 55 kD protease does not bind DEAE, gelatin, heparin, or actin, and plasmin in not potent within the concentration range used here.

The invention may be embodied in other specific forms without departing from it in spirit or essential characteristics. The described embodiments are to be considered in all respects only as illustrative and not restrictive, and the scope of the invention is, therefore, indicated by the appended claims rather than by the above description. All modifications which come within the meaning and range of the lawful equivalency of the claims are to be embraced within their scope.

WHAT IS CLAIMED IS:

1. A composition consisting essentially of an effective, glial cell proliferation-inhibiting concentration of a glial antiproliferative protein, wherein said protein is a NAP of approximately 55 kD having a protease activity.

2. A pharmaceutical preparation, comprising an effective concentration of the glial antiproliferative protein of Claim 1 in a pharmaceutically acceptable carrier.

3. The preparation of Claim 2 wherein said carrier is a biodegradable matrix.

4. The composition or preparation of Claim 1 or Claim 2, wherein said protein is isolated from glial cell culture conditioned media.

5. The composition or preparation of Claim 1 or Claim 2 wherein said protein is a glial cell autocrine protein.

6. The composition or preparation of Claim 1 or 2 wherein said protein has a calcium-dependent, Zn++-containing metalloenzyme activity.

7. A method for isolating a glial antiproliferative peptide sequence from fibronectin, comprising contacting fibronectin with the glial antiproliferative protein of Claim 1.

8. A polypeptide sequence isolated from fibronectin by the method of Claim 7.

9. A polypeptide sequence, comprising at least a portion of the sequence of the polypeptide of Claim 8, and having a neural antiproliferative activity.

10. The polypeptide sequence of Claim 8 or 9 having a molecular weight of approximately 30 kDa.

11. A pharmaceutical preparation, comprising the composition of Claim 8 or 9 in a pharmaceutically acceptable carrier.

12. A preparation according to Claim 11 wherein said carrier is a biodegradable matrix.

13. A composition for inhibiting the growth of glial cells *in vivo*, comprising an effective, growth-inhibiting

amount of the NAP of Claim 1 in a bioerodable matrix providing controlled release of said NAP in vivo.

14. A method for inhibiting the proliferation of glial cells, comprising administering to said cells an effective proliferation-inhibiting amount of NAP.

15. The method of Claim 14, wherein said glial cells are in vivo.

16. The method of Claim 15, wherein said glial cells are associated with injured nervous system tissue.

17. The method of Claim 15, wherein said glial cells are in the central nervous system.

18. A method of treating glial tumors in a patient, comprising applying to the site of said tumor an effective glial cell antiproliferative amount of an agent selected from the compositions of any one of Claims 1, 8, or 9.

19. A method of preventing glial scarring following injury to nervous tissue in a patient, comprising applying to the site of said injury an effective amount of a neural antiproliferative agent selected from the compositions of Claims 1, 8, or 9.

20. Antibodies having a specificity for at least a portion of the sequence of a protein of any one of Claims 1, 8, or 9, and capable of binding thereto.

21. A method of treating demyelinating disease in a patient requiring such treatment, comprising administering to the affected demyelinated lesions of the nervous system of said patient an effective glial cell proliferation promoting amount of the antibodies of Claim 20.

FIG. 1

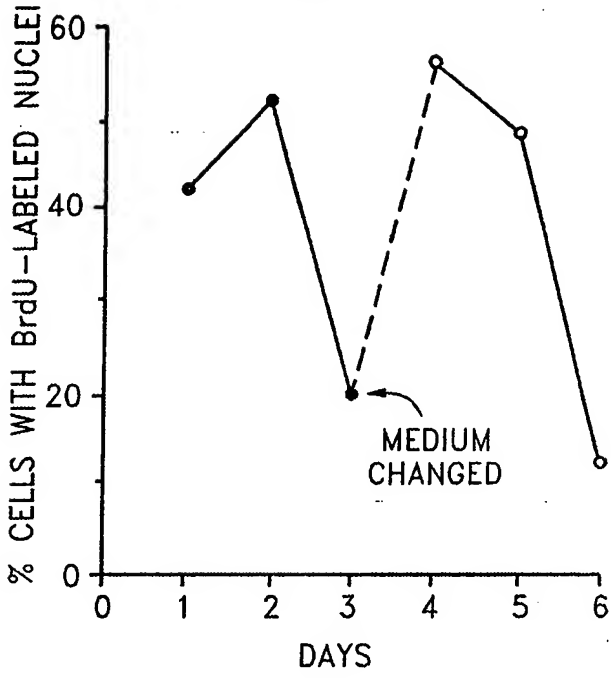
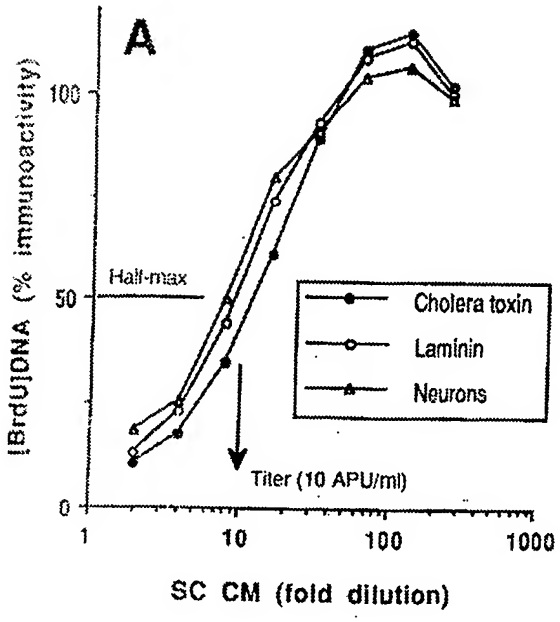


FIG. 2A

FIG. 2B



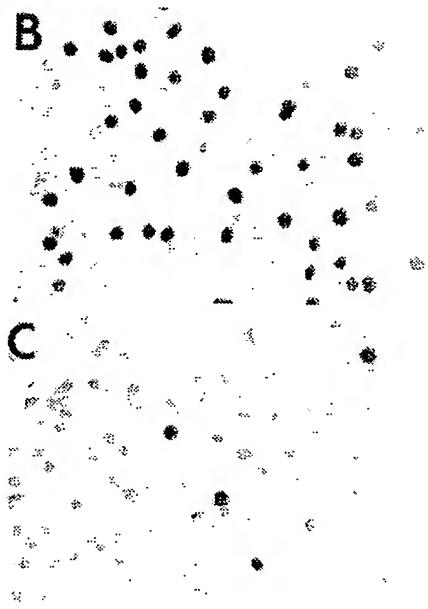


FIG. 2C

FIG. 3A

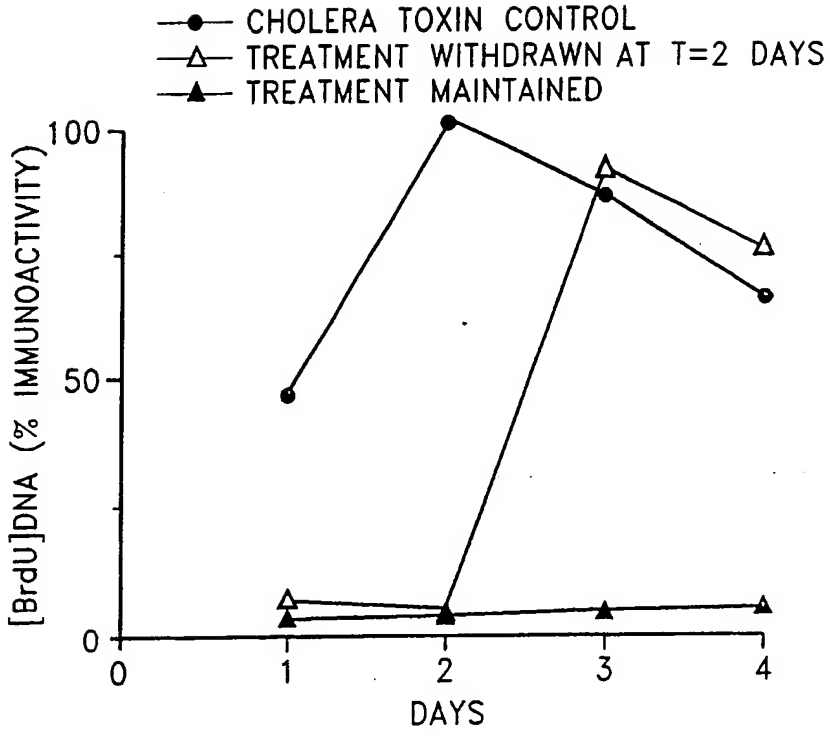

CHOLERA TOXIN CONTROL
TREATMENT WITHDRAWN AT T=2 DAYS
TREATMENT MAINTAINED
[BrdU]DNA (% IMMUNOACTIVITY)
100
50
0
0
1
2
3
4
DAYS

FIG. 3B

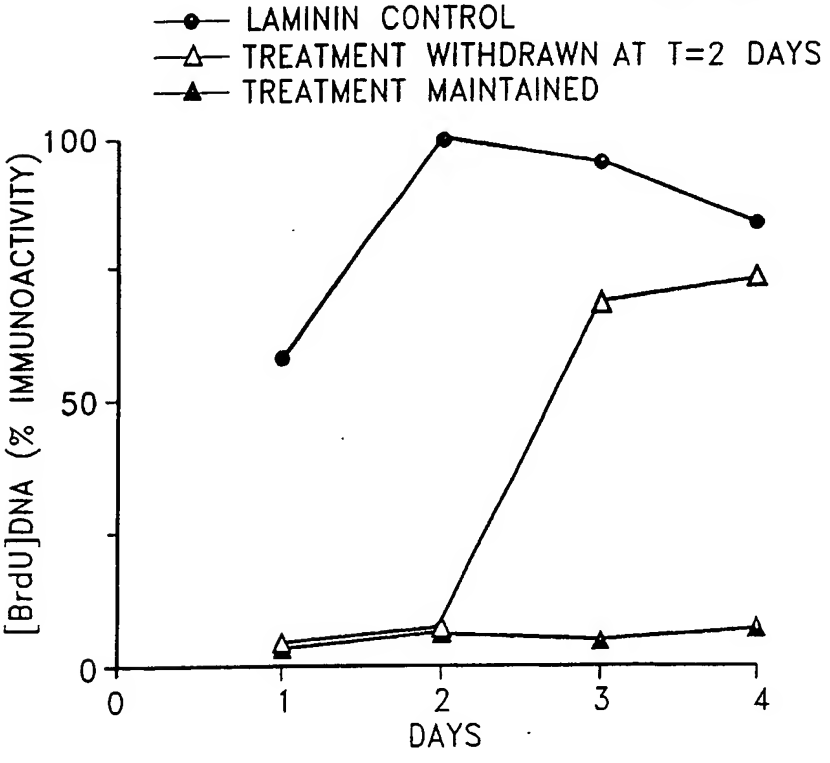

LAMININ CONTROL
TREATMENT WITHDRAWN AT T=2 DAYS
TREATMENT MAINTAINED
[BrdU]DNA (% IMMUNOACTIVITY)
100
50
0
0
1
2
3
4
DAYS

FIG. 3C

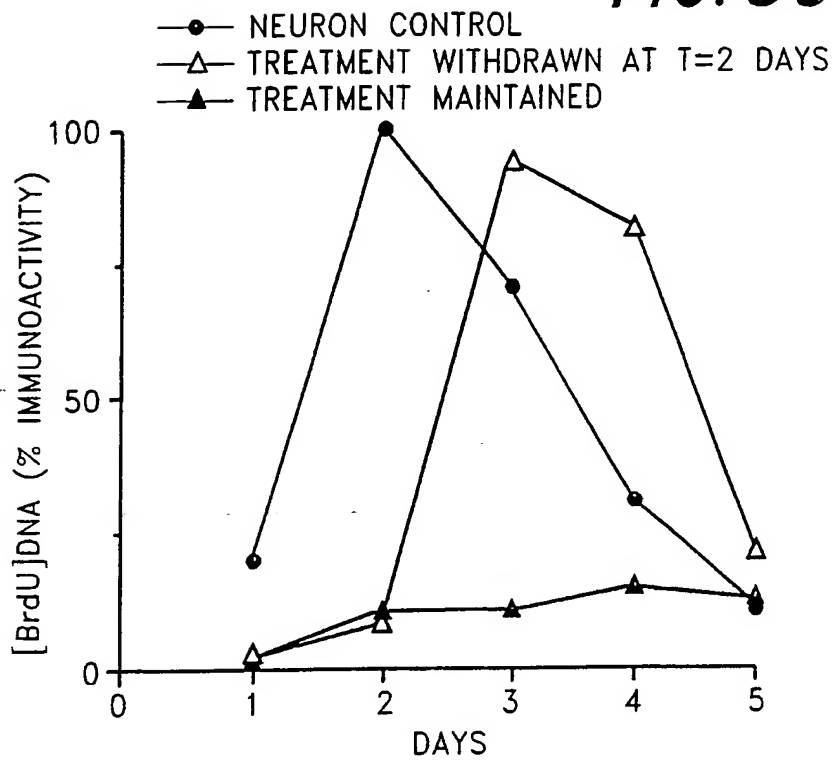

NEURON CONTROL
TREATMENT WITHDRAWN AT T=2 DAYS
TREATMENT MAINTAINED
[BrdU]DNA (% IMMUNOACTIVITY)
100
50
0
0
1
2
3
4
5
DAYS

FIG. 4

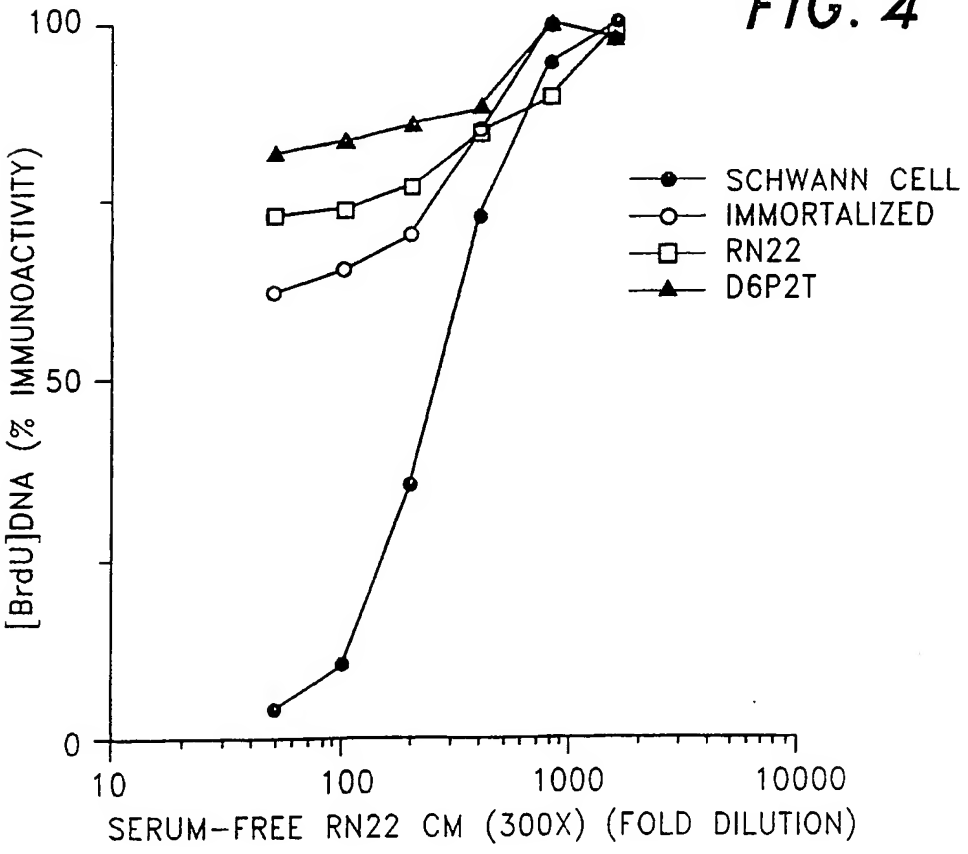

SCHWANN CELL
IMMORTALIZED
RN22
D6P2T
[BrdU]DNA (% IMMUNOACTIVITY)
100
50
0
10
100
1000
10000
SERUM-FREE RN22 CM (300X) (FOLD DILUTION)

FIG. 5A

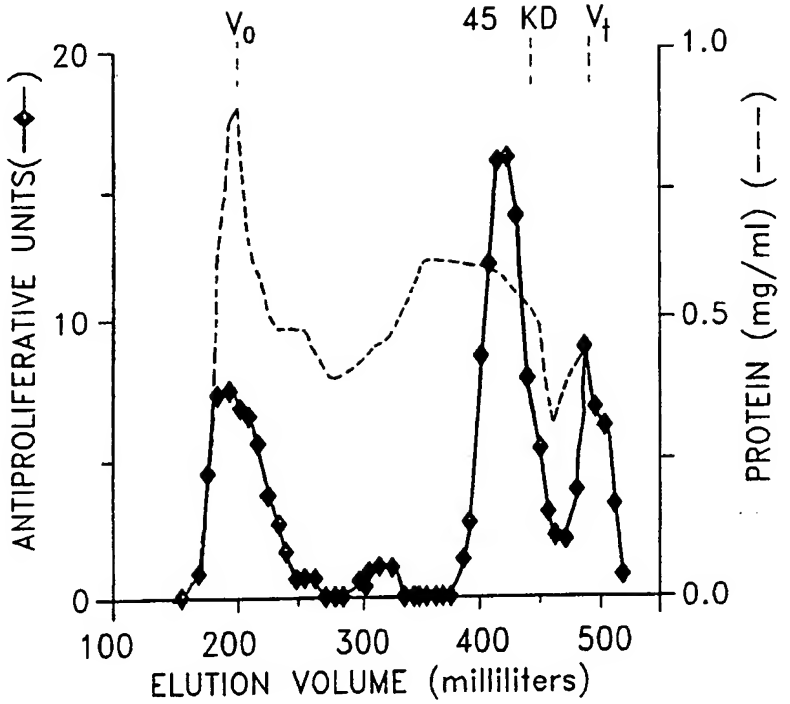

V_0
45 KD
V_t
20
10
0
1.0
0.5
0.0
ANTIPROLIFERATIVE UNITS(—◆—)
PROTEIN (mg/ml) (---)
100
200
300
400
500
ELUTION VOLUME (milliliters)

FIG. 5B

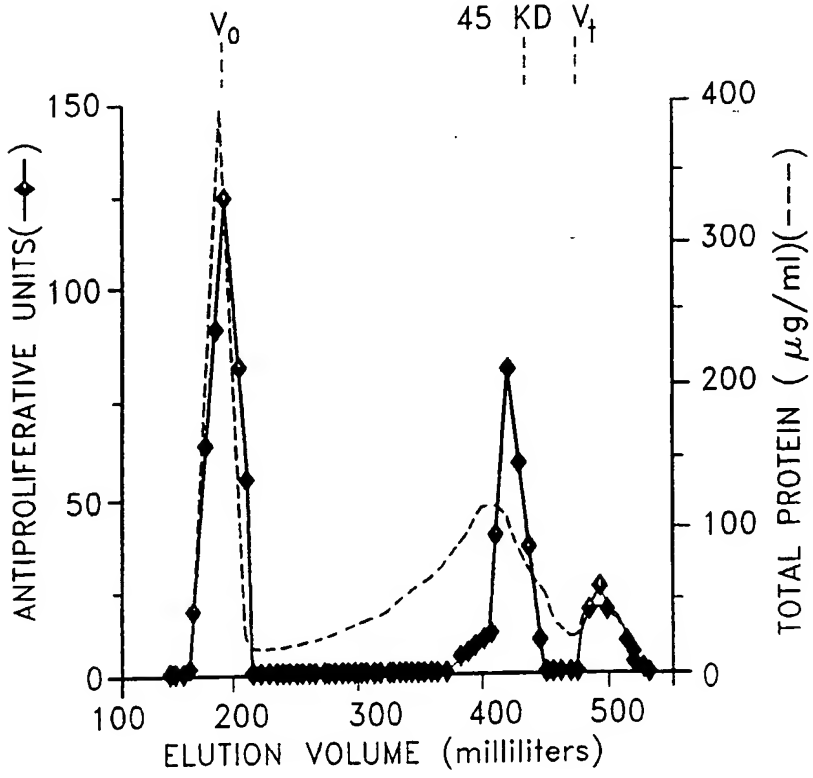

V_0
45 KD
V_t
150
100
50
0
400
300
200
100
0
ANTIPROLIFERATIVE UNITS(—◆—)
TOTAL PROTEIN (μg/ml)(---)
100
200
300
400
500
ELUTION VOLUME (milliliters)

FIG. 5C

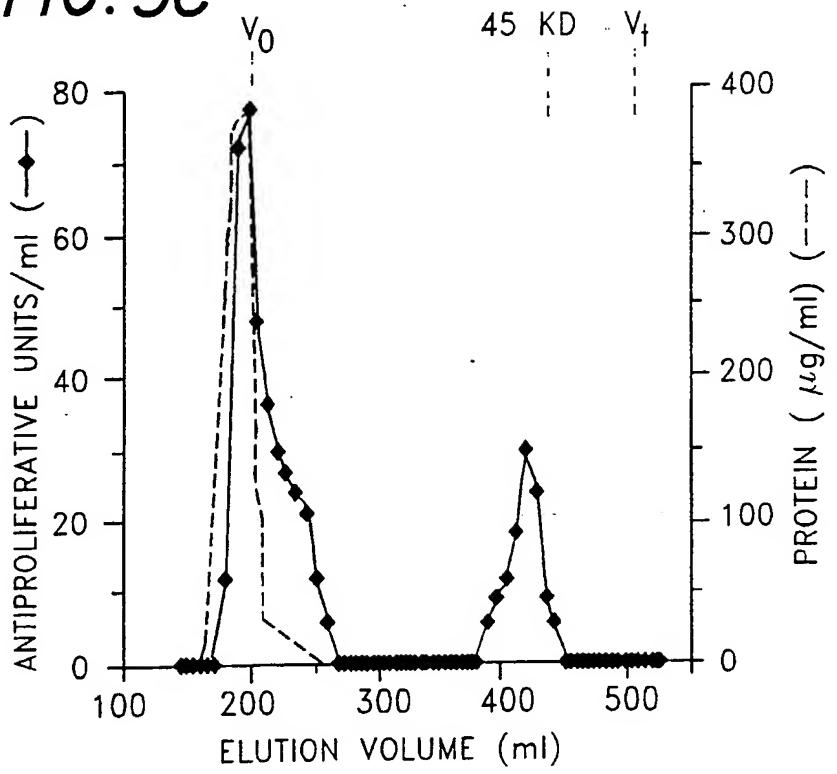

V_0
45 KD
V_t
80
60
40
20
0
400
300
200
100
0
ANTIPROLIFERATIVE UNITS/ml (—●—)
PROTEIN (μg/ml) (---)
100
200
300
400
500
ELUTION VOLUME (ml)

FIG. 5D

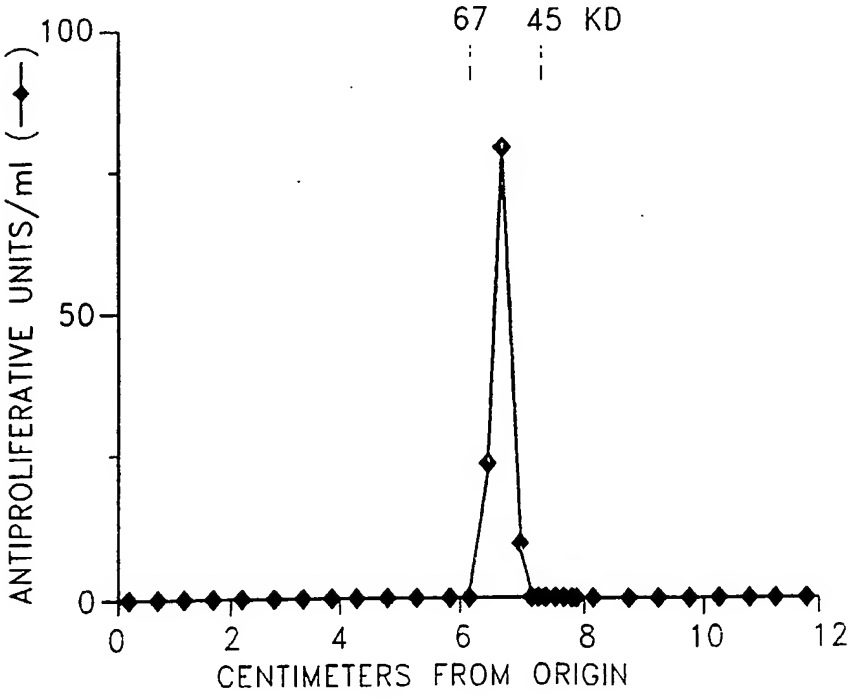

67
45 KD
100
50
0
ANTIPROLIFERATIVE UNITS/ml (—●—)
0
2
4
6
8
10
12
CENTIMETERS FROM ORIGIN

FIG. 6

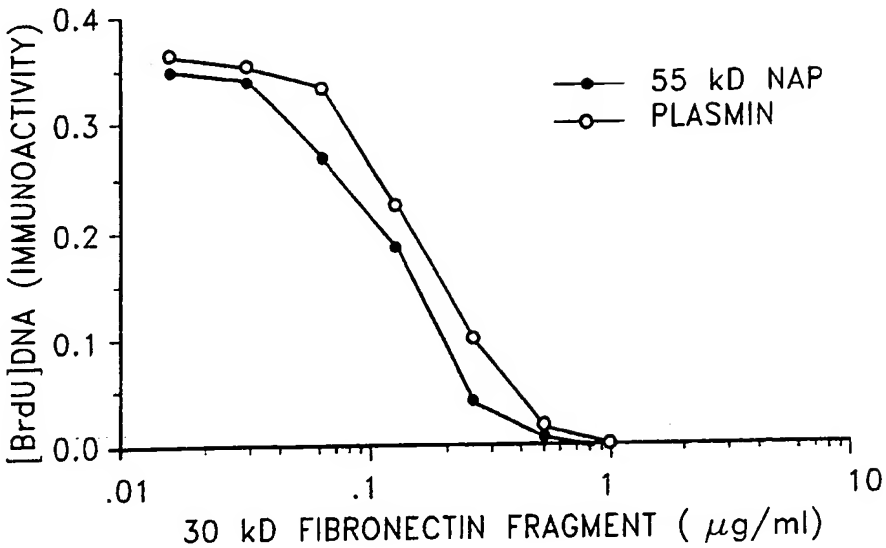
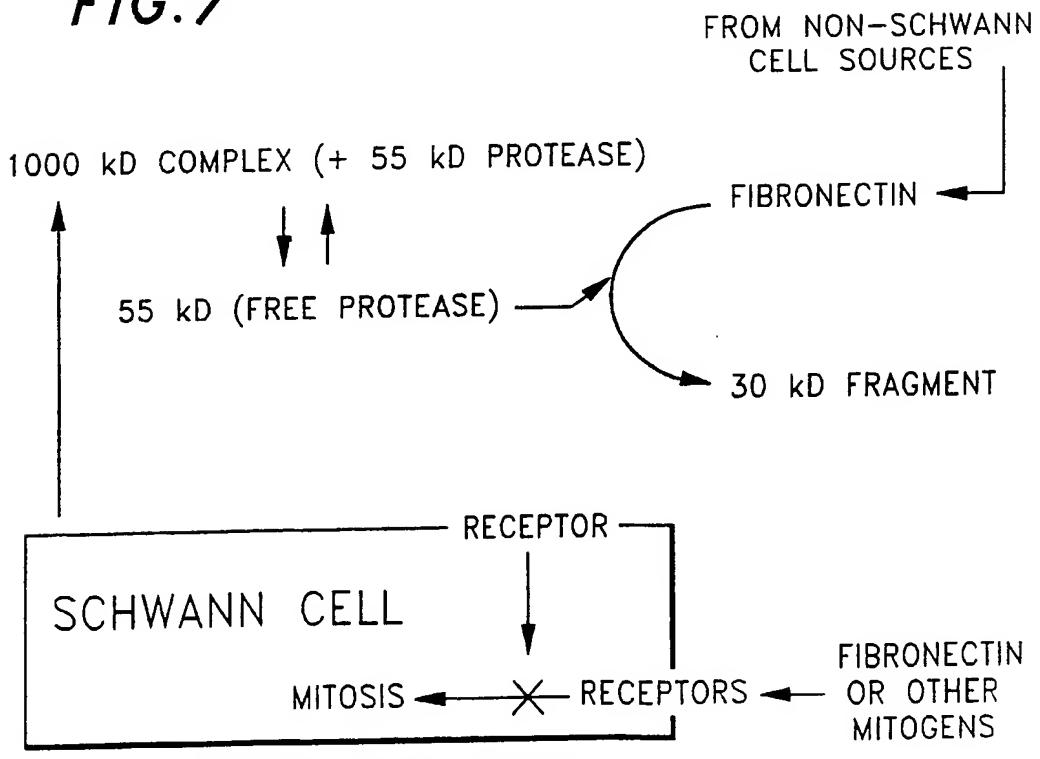

0.4
0.3
0.2
0.1
0.0
[BrdU]DNA (IMMUNOACTIVITY)
55 kD NAP
PLASMIN
.01
.1
1
10
30 kD FIBRONECTIN FRAGMENT (μg/ml)

FIG. 7


FROM NON-SCHWANN CELL SOURCES
1000 kD COMPLEX (+ 55 kD PROTEASE)
FIBRONECTIN
55 kD (FREE PROTEASE)
30 kD FRAGMENT
RECEPTOR
SCHWANN CELL
MITOSIS
RECEPTORS
FIBRONECTIN OR OTHER MITOGENS

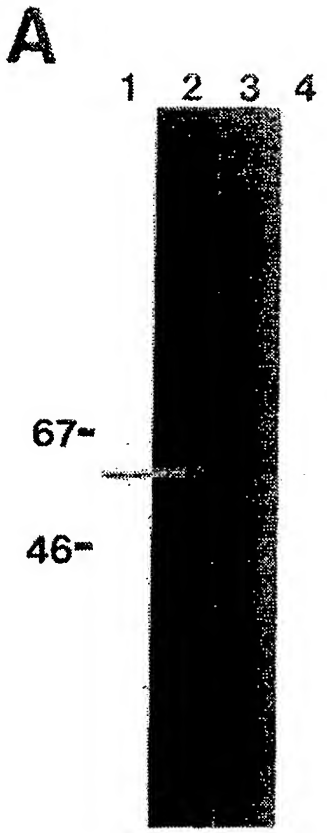


FIG. 8A

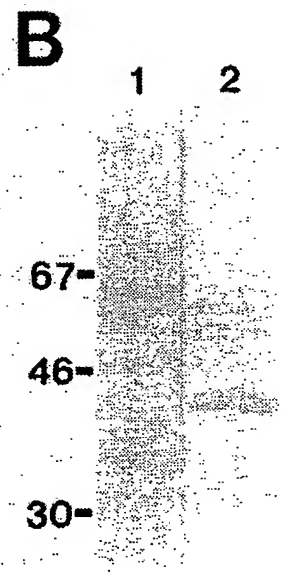


FIG. 8B

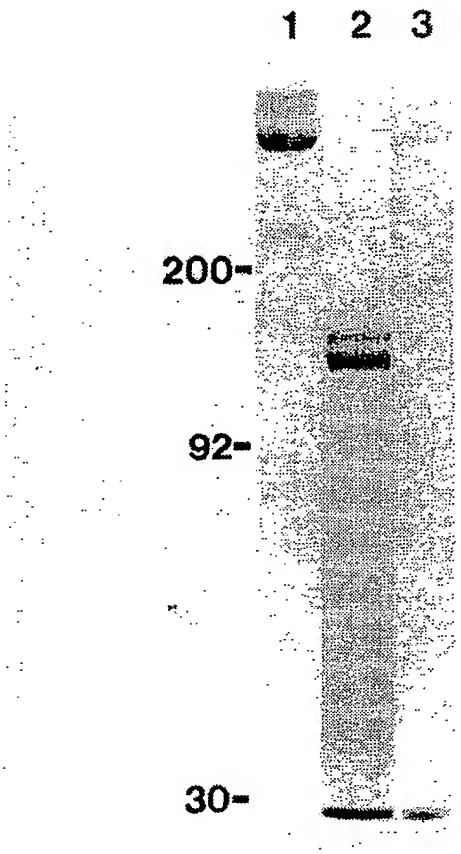
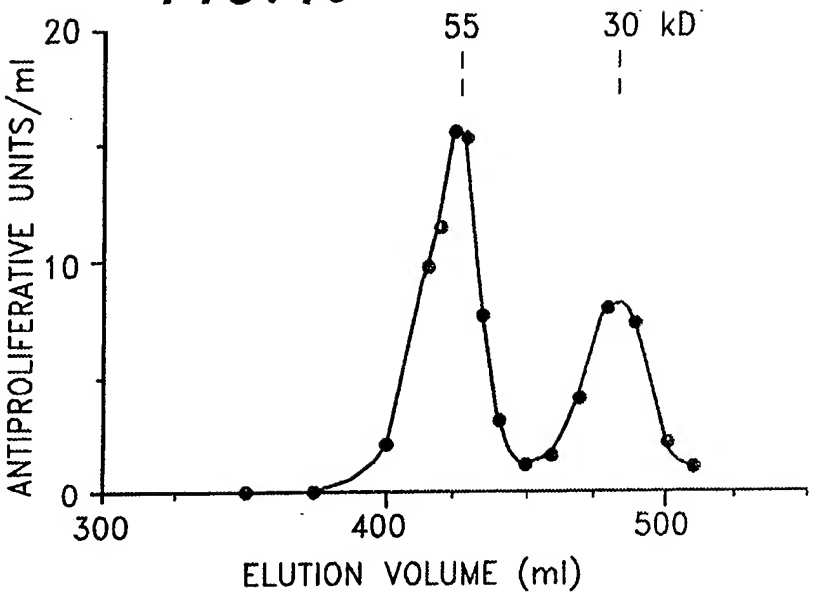


FIG. 9

FIG. 10


55
30 kD
20
10
0
ANTIPROLIFERATIVE UNITS/ml
300
400
500
ELUTION VOLUME (ml)

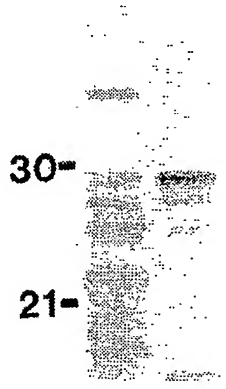
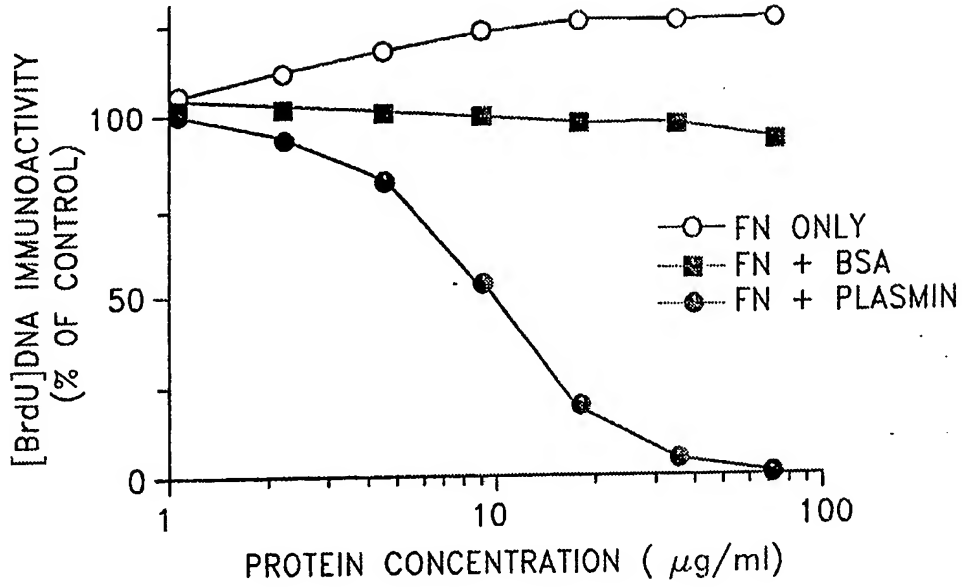

1 2
30-
21-

FIG. 11

FIG. 12


[BrdU]DNA IMMUNOACTIVITY
(% OF CONTROL)
100
50
0
1
10
100
PROTEIN CONCENTRATION (μg/ml)
FN ONLY
FN + BSA
FN + PLASMIN

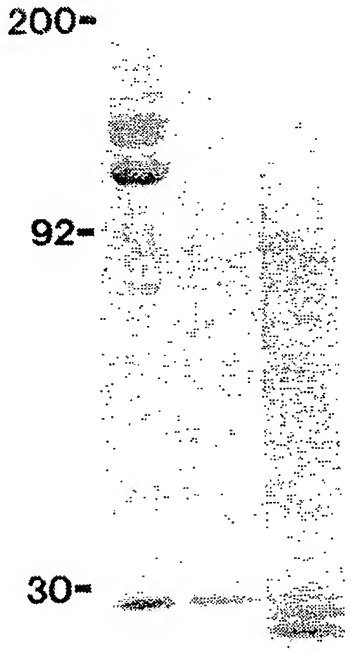

1 2 3
200-
92-
30-

FIG. 13

FIG. 14A

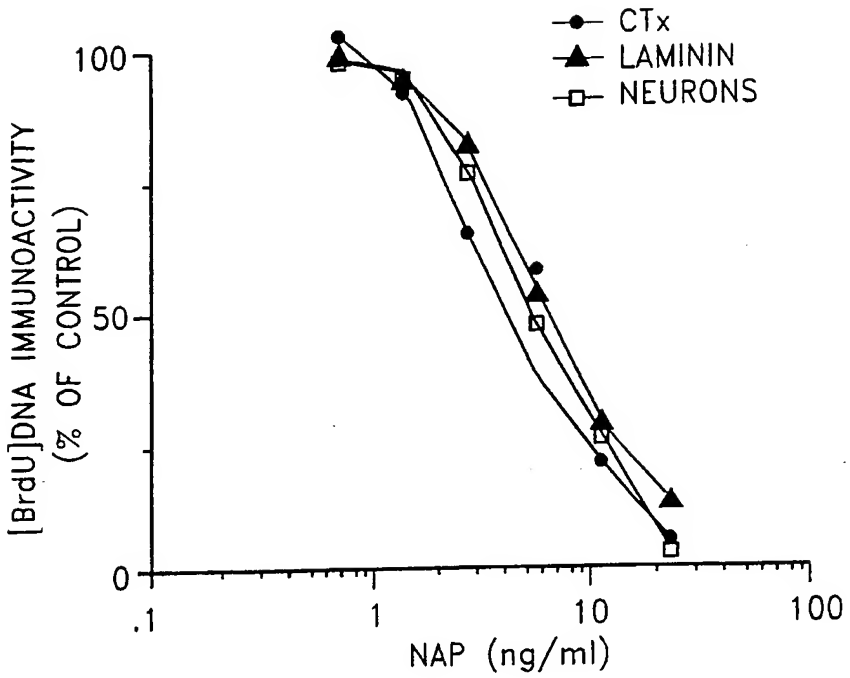


FIG. 14B

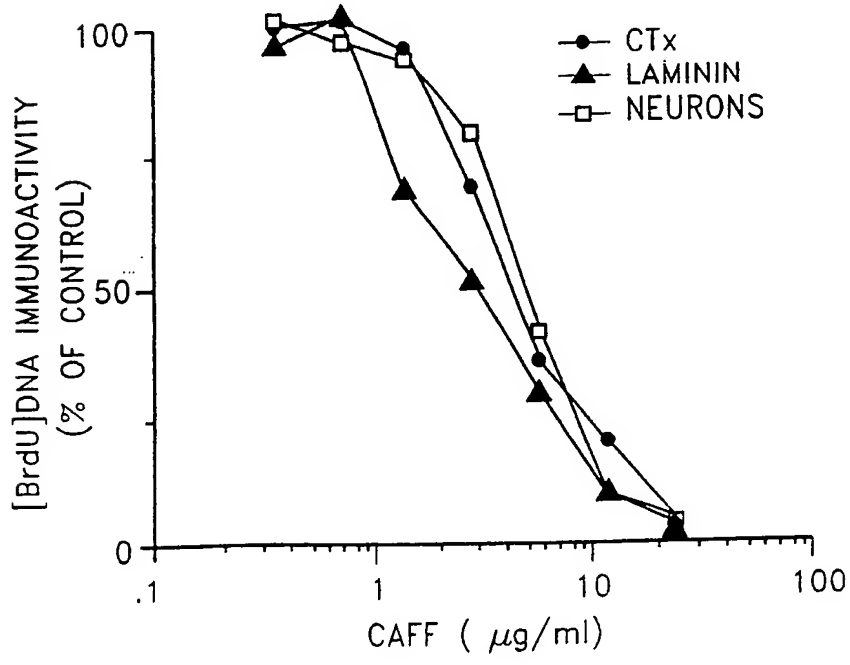


FIG. 14C

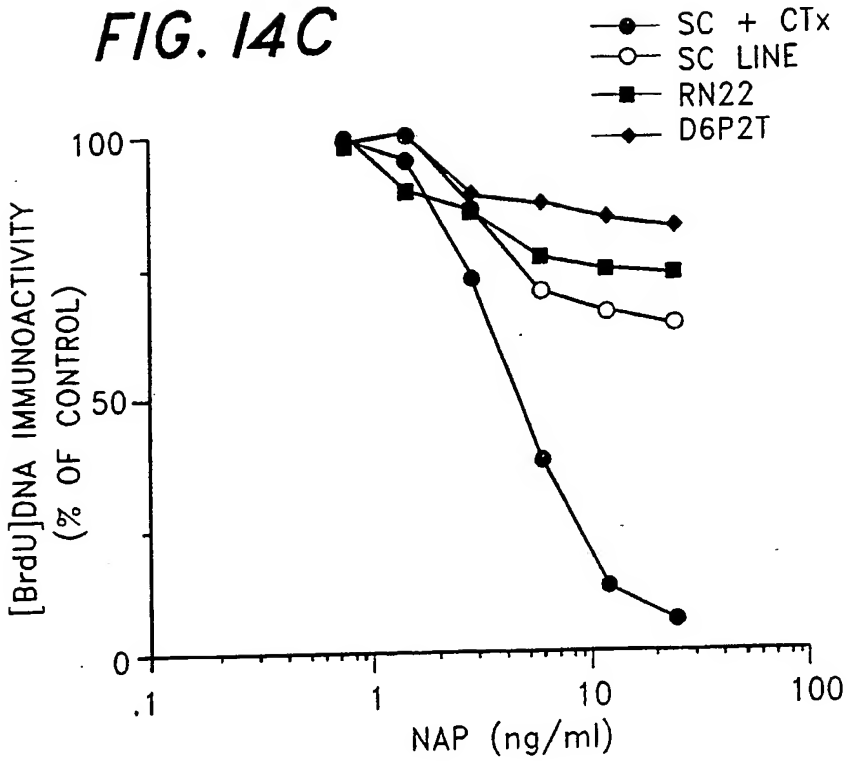
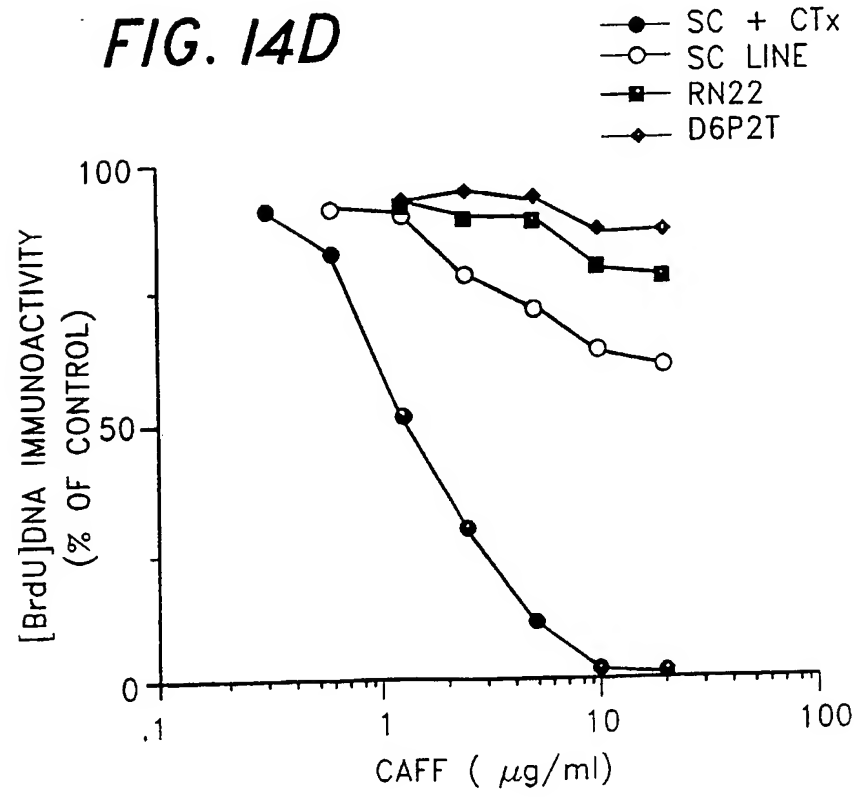


FIG. 14D



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/06476

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 9/50; A61K 37/54

US CL: 435/219; 424/94.67

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US Cl.	435/219; 424/94.67

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS BIOSIS
CAS MEDLIN

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	CHEMICAL ABSTRACTS. Volume 103. No. 5. issued 05 August 1985, Pittman et al.. "Releases of Plasminogen Activator and A Calcium-Dependent Metalloprotease from Cultured Sympathetic and Sen sory Neruons", see page 314, Abstract No. 35222. Dev. Bio., 110(1). 91-101.	1-6.13.14-19 1-6.13.14-19
X Y	CHEMICAL ABSTRACTS, Volume 112. No. 11. issued 12 March 1990. Case et al. "Transin/Stromelysin Expression in Rheumatoid Synovium. A Transformation-Associated Metalloproteinase Secreted by Phenotypically Invasive Synoviocytes", see page 554. Abstract No. 96395. Am. J. Pathol. 135 (6), 1055-1064.	1-6.13.14-19 1-6.13.14-19

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
23 DECEMBER 1991	10 JAN 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/USA	JON P. WEBER

Form PCT/ISA/210 (second sheet) (Rev.11-87)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X Y	CHEMICAL ABSTRACTS, Volume 109, No. 21, issued 21 November 1988. Zucker et al., "Characterization of a Connective Tissue Degrading Metalloproteinase from Human Small Cell Lung Cancer Cells", see page 308. Abstract No. 185997. Clin. Exp. Metastasis. 6 (5), 363-375.	1-6,13,14-19 1-6,13,14-19
X Y ,P	Chemical Abstracts. Volume 115, No. 1. issued 08 July 1991, Quigley et al., "Serine Protease and Metallo Protease Cascade Systems Involved in Pericellular Proteolysis". see page 600. Abstreact No. 6056, Cell Differ Dev., 32(3), 263-275.	1-6,13,14-19 1-6,13,14-19
X Y	CHEMICAL ABSTRACTS. Volume 106. No. 11. issued 16 March 1987, Rutka et al., "Effects of Extracellular Matrix Proteins on the Growth and Differentiation of an Anaplastic Glioma Cell Line". see page 409. Abstract No. 82370. Can. J. Neurol. Sci., 13(4). 301-306.	1-6,13,14-19 1-6,13,14-19
X Y	CHEMICAL ABSTRACTS. Volume 113. No. 5, issued 30 July 1990, Chantry et al., "A Novel Metalloproteinase Originally Isolated from Brain Myelin Membranes is Present in Many Tissues". see page 356, Abstract No. 3567, Biochem. J., 268 (1).245-248.	1-6,13,14-19 1-6,13,14-19
X Y	CANCER RES., Volume 47. No. 13. issued 01 July 1987, Rutka et al., "Inhibition of Growth and Induction of Differential in a Malignant Human Glioma Cell Line by Normal Leptomeningeal Extracellular Matrix Proteins", pages 3515 -3522. see entire document.	1-6,13,14-19 1-6,13,14-19
X Y	CANCER RES., Volume 50. No. 8. issued 15 April 1990, Apodaca et al., "Expression of Metalloproteinases and Metalloproteinase Inhibitors by Fetal Astrocytes and Glioma Cells". pages 2322 -2329, see entire document.	1-6,13,14-19 1-6,13,14-19

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	Astrocytes and Glioma Cells", pages	
A	THROMB. RES.. Volume 33. issued 1984. Wojtechka-Lukasik et al.. "Effects of Neutral Proteases from Human Leukocytes on Plasea Fibronectin". pages 471-476. see entire document.	1-6.13.14-19
Y	BIOCHEMISTRY. Volume 17. No. 12. issued June 1978. Stricklin et al.. "Human Skin Fibroblast Collagenase: Chemical Properties of Precursor and Active Forms", pages 2331-2337. see entire document.	1-6.13.14-19
X Y	US.A, 4,923,818 (GOLDBERGET AL.) 08 MAY 1990. see especially column 8. lines 9-36. and column 12. lines 12-22.	1-6.13.14-19 1-6.13.14-19